Cape Peninsula
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Cultivation and identification of filamentous bacteria from twelve wastewater treatment plants in South Africa

By

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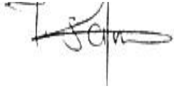

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ABSTRACT

The activated sludge process is the most extensively utilized technology for domestic wastewater treatment. Due to the increasing demand for clean water supply and proper environmental management, engineers and scientists are constantly looking for ways to improve process efficiency. Since the introduction of the activated sludge process, the most prevalent operational problems reported are sludge bulking and foaming caused by over-proliferation of filamentous bacteria. Attempts have been made to resolve filamentous bulking and foaming, but short-term control strategies are often ineffective long-term. In order to gain further insight into bulking and foaming, a deeper understanding of the associated filamentous bacterial populations is required. In previous decades, the unreliability of traditional (light microscopy and culture-dependent) microbiological methods has impeded the identification of filamentous bacteria and the study of their physiology. However, knowledge gaps regarding filamentous bacteria identity and function at a molecular level still exist.

Therefore, this study was aimed at identifying filamentous bacterial populations found in wastewater treatment plants in South Africa by means of both conventional (phenotypic) and molecular (phylogenetic) identification methods. Filamentous bacteria were isolated from mixed liquor samples taken from wastewater treatment plants with histories of bulking and foaming. Deoxyribonucleic acid (DNA) was extracted from the isolates and amplified via the polymerase chain reaction using universal primers, after which the amplicons were sequenced. The overall bacterial community structures in the genomic DNA extracted from the activated sludge from the selected study sites were determined using amplicon sequencing (Illumina MiSeq). Results were compared with historical results obtained using classical light-microscopy.

Twelve isolates which exhibited filamentous forms were cultured. Four of eight isolates from which high-quality DNA was extracted continuously exhibited cellular morphology typical of filamentous bacteria throughout the study, while the remainder shifted from filamentous to single cell forms upon repeated sub-culturing. Sixty percent of the isolates were members of the class *Gammaproteobacteria,* while 40% were members of the class *Bacillus,* neither of which have previously been associated with Eikelboom filamentous morphotypes.

Amplicon sequencing revealed that *Lewinella* spp, *Sphaerotilus* spp, *Haliscomenobacter* spp, *Tetrasphaera* spp, *Fluviicola* spp, *Longilinea* spp, *Bellilinea* spp, *Crocinitomix* spp, and *Mycobacterium* spp, were the most dominant filamentous bacteria that were not identified via light microscopy. Comparison of light microscopy

and amplicon sequencing results for filament identification revealed notable differences and highlighted the difficulties associated with both methods. This study contributes to the body of knowledge on filament identification in the activated sludge of wastewater treatment plants from different geographical locations in South Africa. The NGS findings from this study showed that there are many filamentous bacteria dominating the activated sludge plants and may be playing crucial roles which have not yet been studied and characterised. Therefore, this study can be used as a basis to gain further knowledge on the phylogeny of the filamentous bacteria community especially in the South African context.

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DEDICATION

This thesis is dedicated to:

My family both living and dead

To my late grandmother **Nofenishala Sam**

My mother **Nomfundo Sam** (Pillar of strength)

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ABBREVIATIONS

AS	Activated sludge
ASP	Activated sludge process
BNR	Biological nutrient removal
BOD	Biological oxygen demand
DO	Dissolved oxygen
EBNR	Enhanced biological nutrient removal
EBPR	Enhanced biological phosphorus removal
F/M	Food to microorganisms
MLE	Modified Ludzack-Ettinger
MLR	Mixed liquor recycle
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NGS	Next generation sequencing
PAOs	Phosphate accumulating organisms
PCR	Polymerase chain reaction
PHAs	Polyhydroxyalkanoates
RAS	Return activated sludge
SVI	Sludge volume index
UASB	Up flow anaerobic sludge blanket
VFAs	Volatile fatty acids
WAS	Waste activated sludge
NOB	Nitrite oxidising bacteria
WWTWs	Wastewater treatment works
AOB	Ammonium oxidising bacteria
UCT	The University of Cape Town
EF	Effluent
IF	Influent
EPS	Extracellular polymeric substances
H_2O_2	Hydrogen peroxide
T-RFLP	Terminal restriction fragment length polymorphism

CHAPTER 1
INTRODUCTION

1.1. Background

South Africa is the 30th driest country worldwide (Kohler, 2016). The increasing water shortage is a consequence of rapid population growth and industrial and economic development. Due to large quantities of wastewater being produced from a variety of human activities, efficient wastewater treatment has become very important (Gupta *et al.*, 2012; Wei *et al.*, 2012; Suárez-Varela *et al.*, 2013; Teklehaimanot *et al.*, 2015). Discharging wastewater that has not been properly treated may result in contamination of water bodies by sewage, which pollutes the environment and poses a risk to human health (Agrawal *et al.*, 2010; Mema, 2010; Abdel-Raouf *et al.*, 2012; Naidoo and Olaniran, 2014). Wastewater treatment processes such as the activated sludge process (ASP) is used to prevent contamination of water bodies by organic matter, pathogens and other pollutants present in domestic and industrial wastewater (Nourmohammadi *et al.*, 2013).

The ASP is a biological wastewater treatment process (Hait and Mazumder, 2011; Khairnar *et al.*, 2014) that exploits the ability of a complex consortium of macro- and microorganisms to degrade soluble and insoluble organic matter and convert this matter into a flocculent microbial suspension that settles well in a conventional clarifier (Ramothokang *et al.*, 2003; Martins *et al.*, 2004; Marrengane, 2007). In this complex community, bacteria play a key role and account for about 95% of removal functions (Martins *et al.*, 2004; Juang and Chiou, 2007). Filamentous and other floc-forming bacteria are particularly important (Madoni *et al.*, 2000; Larsen *et al.*, 2008). Filamentous bacteria are essential constituents of healthy activated sludge and are necessary for proper floc formation and settling of solids (Séka *et al.*, 2003; Yang *et al.*, 2011). Under optimum conditions, floc-forming bacteria predominate resulting in a good sludge settling (Naidoo, 2005). The composition of wastewater treatment plants changes continuously and can trigger an overgrowth of filamentous bacteria that do not form part of the flocs or extend from the flocs and form bridges between flocs. This hampers settling, and is known as filamentous bulking (Caravelli *et al.*, 2003; Khairnar *et al.*, 2014; Lou and Ieong, 2015). In addition, overgrowth of certain filamentous bacteria with hydrophobic properties can also be triggered, promoting formation of stable foam that may pass into the activated sludge effluent resulting in an increased biological oxygen demand (BOD) and suspended solids in the effluent, known as filamentous foaming (Wagner *et al.*, 2002; Griffiths *et al.*, 2010). A major advantage of

the ASP compared to other processes is its flexibility with regards to process configurations (Vasquez Sarria *et al.*, 2011; Aguilar-López *et al.*, 2013). This process can be modified according to the influent type and required effluent standards (Naidoo, 2005; Rustum, 2009). The majority of ASPs have been configured so as to facilitate Biological Nutrient Removal (BNR). BNR refers to biological removal of carbon, nitrogen (through nitrification/de-nitrification process) and in some cases, phosphorus, through the action of phosphorus-accumulating organisms (Aguilar-López *et al.,* 2013; Saunders *et al.,* 2015).

Despite the fact that the ASP has been utilised for decades in many countries including South Africa, a significant number of treatment plants continue to encounter intermittent or sustained settling problems (Ahansazan *et al.*, 2014). Prevention of filamentous bulking and foaming is challenging, despite extensive research devoted to this topic (Khairnar *et al.*, 2014). To counter filamentous bulking and foaming in wastewater treatment facilities, different types of physical, chemical, and biological methods are utilised. However, none of the current methods available are completely effective (Mamais *et al.*, 1998; Séka *et al.*, 2003; Liu *et al.*, 2016). For example, biocides used to suppress the growth of filamentous bacteria are non-specific and can also have a detrimental effect on other functional members of the sludge community such as floc-formers and nitrifiers (Guo *et al.*, 2012).

An alternative treatment approach would be the use of bacteriophages; these are viruses that are ubiquitous in the environment, being found in association with their bacterial hosts (Khan *et al.*, 2002; Clokie *et al.*, 2011; Keary *et al.*, 2013; Aziz *et al.*, 2015) and can potentially be used as host specific, environmental friendly and cost effective biological agents to control bulking and foaming in activated sludge systems (Liu *et al.*, 2015). Bacteriophages have been detected in wastewater systems; however, their role in the microbial community is poorly understood (Khan *et al.*, 2002). Since their discovery, their bactericidal functions have drawn much interest (Withey *et al.*, 2005; Rattanachaikunsopon and Phumkhachorn, 2007). Bacteriophages possess properties that make them excellent candidates as therapeutic or bio-control agents. These include specificity, adaptability, the ability to self-replicate, effectiveness in killing target bacteria, and the fact that they are naturally resident in the environment (Jassim *et al.*, 2016). Although the use of bacteriophages shows potential as an environmentally friendly, host specific and cost effect control method for bulking and foaming, very little is known about their diversity and impact of their activity in full scale systems (Runa *et al.*, 2021). Therefore, they remain an unexplored technology for controlling bulking and foaming sludge in full-scale plants (Kotay *et al.*, 2013)

It is well established that a thorough knowledge of the bacterial populations in wastewater treatment works (WWTWs) is required to reveal factors that impact the efficiency and stability of biological treatment processes such as ASP so as to develop suitable strategies for improved process performance. Traditionally, bacterial populations in WWTWs have been studied either by microscopic observation or by culture-dependent techniques. However, only few filamentous bacteria can be identified reliably based on morphological features. Therefore, this necessitates the use of a holistic approach using both conventional and molecular techniques for identification.

1.2. Problem statement and aim of study

The ASP is the most widely used technology for domestic wastewater treatment (Withey *et al.*, 2005; Mielczarek *et al.*, 2013). The most prevalent operational problems reported in ASP are sludge bulking and foaming caused by over-proliferation of filamentous bacteria (Madoni *et al.*, 2000; Liu and Liu, 2006). At present, there's no universal strategy to control bulking and foaming (Choi *et al.*, 2011; Khairnar *et al.*, 2014; Liu *et al.*, 2015). Therefore, this study was aimed at identifying filamentous bacterial populations found in wastewater treatment plants in South Africa by means of both conventional (phenotypic) and molecular (phylogenetic) identification methods.

1.3. Research objectives

The following objectives were identified**:**

1. To confirm the previously identified most dominant filamentous bacteria in activated sludge samples using Next Generation Sequencing (NGS).
2. To isolate filamentous bacteria implicated in activated sludge bulking and foaming.
3. To confirm the identification of the isolated filamentous bacteria by sequencing 16S rRNA gene amplicons (PCR-based).
4. To screen activated sludge samples for the presence of bacteriophages capable of infecting the filamentous isolates.

1.4. Specific research questions

Objective 1

- What are the most dominant phyla to which filamentous bacteria belong to? Do geographic location, configurations and seasonal variation have an impact on their dominance?
- Are there other abundant filamentous bacteria that may have been missed by microscopic identification?

Objective 2

- Can filamentous bacteria implicated in activated sludge bulking and foaming be isolated?

Objective 3

- Are there co-occurring bacteriophages that can infect these isolated filamentous bacteria?

Objective 4

- What are the phylogenetic names of the isolated species?

1.5. Significance of the study

The prevalence of filamentous bulking and foaming in activated sludge wastewater treatment plants has been widely reported worldwide, including in South Africa. Currently there's no effective control method; they are either short term or not effective at all. This is due to the fact that the filamentous bacterial populations in ASPs are poorly understood which could be due to problems of cultivation because of their slow growing nature and incorrect microscopic identification. To date, no studies have been conducted on the identification of filamentous bacteria from wastewater ASPs in South Africa using both conventional and molecular methods. This current study will add to the body of knowledge for the development of an efficient, cost effective and environmentally friendly method to control bulking and foaming sludge. In addition, controlling bulking and/or foaming will improve the quality of treated domestic effluent, ensure better compliance with regulatory municipal discharge standards, and reduce the risk to the environment and human health.

CHAPTER 2
LITERATURE REVIEW

2.1. Wastewater treatment and activated sludge

The activated sludge process (ASP) has become the most widely used process for treatment of both domestic and industrial wastewater (Bafghi and Yousefi, 2016; Lawson, 2018). It is an important and integral part of wastewater treatment plants that treats wastewater either from municipality or industry having soluble organic impurities or a mix of the two types of wastewater sources (Mittal, 2011). Irrespective of the source, wastewater must be treated before being discharged to the receiving water body (Akpor, 2011). In light of many reasons related to social and economic aspects of human life such as public health, environmental protection, and aesthetics, treatment of wastewater is imperative (Naidoo, 2004). Depending on the source, wastewater contains varying amounts of organic matter and/or inorganic particulates, and numerous pathogens. Discharging wastewater containing these pollutants can result in the death of aquatic species, and make the water unsuitable for any human consumption and activities (e.g., swimming and fishing) (Gray *et al.*, 2002). The function of wastewater treatment is to prevent adverse effects on the environment and human health by reducing the concentration of contaminants (Gupta *et al.*, 2012). This is achieved by following a series of steps which involves physical, chemical and biological unit processes (Table 2.1) (Naidoo, 2004).

The requirement for improved treatment methods presents an on-going challenge because increasingly strict effluent quality standards are being introduced (Mahlambi *et al.*, 2015). In addition, restrictions on using certain chemicals and the necessity to reduce costs focus attention on biological treatment processes such as the ASP (Meijer, 2004; Wan *et al.*, 2016). Biological processes are very important and integral parts of wastewater treatment, and can be used to treat either domestic or industrial wastewater or a mixture of wastewater from both sources (Sinha *et al.*, 2014). They are classified as aerobic or anaerobic processes based on the oxygen dependence of primary microorganisms responsible for the treatment. In aerobic processes, aerobic and facultative microorganisms are responsible for treatment while in anaerobic processes, anaerobic and facultative microorganisms are responsible for treatment (Burgess and Pletschke, 2008; Hung *et al.*, 2012).

Table 2.1: Typical wastewater treatment steps (Adapted from Metcalf and Eddy, 1991, as cited in Zickefoose, 2004)

	Description	References
Physical screening (primary treatment)	Wastewater influent is passed through mechanically raked bar screens to remove large objects (rags, sticks, toilet paper, grit, and grease) that may damage the pumps or clog the pipes.	Cheremisinoff, 2002; Gray *et al.*, 2002; Abdel-Raouf *et al.*, 2012
Primary settling (physical treatment)	Primary settling removes smaller suspended solids and colloidal particles by physical settling (sedimentation) in tanks. Its goal is to produce a liquid effluent which is suitable for downstream biological treatment.	Tsang *et al.*, 2008; Abdel-Raouf *et al.*, 2012
Biological treatment (secondary treatment)	Uses microorganisms to degrade soluble and insoluble organic matter present in wastewater, thus reducing the biological oxygen demand (BOD). A variety of secondary treatments are available. They are classified as either suspended or attached growth systems.	Abdel-Raouf *et al.*, 2012; Naidoo and Olaniran, 2014
Clarification (secondary settling)	Biological solids (sludge) are settled by gravity. The clear secondary effluent may be disinfected prior to release or may be directly released to the receiving environment.	Ghawi, 2011
Tertiary treatment	Wastewater may not meet discharge or re-use standards for specific water quality parameters after biological treatment. Tertiary treatment includes disinfection, and removal of excess nutrients and suspended solids through coagulation and filtration, respectively.	Cheremisinoff, 2002; Gupta *et al.*, 2012.

Biological processes are further divided into attached and suspended growth systems (Table 2.2) (Cheremisinoff, 2002; Abdulgader *et al.*, 2007; Azizi *et al.*, 2013). The former include trickling filters, rotating biological contactors, biological aerated filters, and fluidized bed biofilm reactors, where microorganisms responsible for the conversion of organic material or nutrients are attached to an inert packing material forming a biofilm (Badireddy *et al.*, 2010; Grady *et al.*, 2011; Dabi, 2015). As the wastewater comes into contact with the biofilm, organic matter is removed and

degraded to produce an acceptable quality effluent (Cheremisinoff, 2002; Shahot *et al.*, 2014).

The main advantages of attached growth systems include low energy requirement, lower sludge production, no problems of sludge bulking in the secondary clarifier, better sludge thickening properties, and low maintenance. The disadvantages include the possibility of odour problems, high BOD and solids concentration in the effluent, as well as problems with biofilm maintenance due to excessive sloughing (Azizi *et al.*, 2013; Dabi, 2015).

In contrast to attached growth systems, suspended growth systems entail that the microbial community is continuously mixed in suspension in wastewater (Bafghi and Yousefi, 2016; Ebrahimi and Najafpour, 2016). Suspended growth systems are primarily used to treat domestic wastewater, removing about 90% of BOD. They are said to be generally less susceptible to disturbances in comparison to attached growth systems. Examples of suspended growth systems include, membrane bioreactors, up flow anaerobic sludge blankets, and oxidation ponds (Karia and Christian, 2006; Krantzberg, 2010), with the ASP being the most widely used (Naidoo, 2005).

Table 2.2: Attached and suspended growth systems used for domestic wastewater treatment

Attached growth systems		
System name	**Operating principle/s**	**Reference/s**
Tricking filters	Organic matter in wastewater is metabolised by microorganisms attached to a medium (filter bed). Wastewater is sprinkled over the filter bed. Aerobic conditions are maintained by diffusion, splashing and either natural draft or forced air. The biological slime thickens and eventually gets detached from the surface (this is called sloughing). A settling tank after the tricking filter removes detached solids.	Pal *et al.*, 2010; Vianna *et al.*, 2012
Rotating Biological Contactors	Uses a similar principle as tricking filters, except that the medium is rotating, and the wastewater is stationary. The rotation of the medium (discs) allows alternating contact of the microbes with the organic matter in wastewater and oxygen in the air thereby maintaining aerobic conditions.	Mba and Bannister, 2007; Cortez *et al.*, 2008; Ghawi, 2009

Table 2.2 Continued

System name	Operating principle/s	Reference/s
Biological Aerated Filters	Wastewater is pumped upwards or downwards and passed through the granular media (filter). Air is diffused upwards through granular media to promote biomass growth. Excess biomass is removed by periodic backwashing of the media bed.	Pramanik *et al.*, 2012
Fluidized Bed Biofilm Reactor	Wastewater is pumped upward through a bed of particles (silica sand) at velocities sufficient to induce fluidisation of the media. Once fluidised, each particle provides a large surface area for biofilm formation and growth. Very dense concentrations of microorganisms growing on the surface of bed particles consume biodegradable waste contaminants in the liquid.	Fuentes *et al.*, 2005; Burghate and Ingole, 2013
Up flow Anaerobic Sludge Blanket (UASB)	Wastewater flowing upwards through the blanket is processed by anaerobic microorganisms leading to the formation of a sludge blanket. The sludge blanket is suspended by settling action of gravity with the aid of flocculants. The clarified effluent is extracted from the top of the tank by a Gas-Liquid-Solid Separator.	Nicolella *et al.*, 2000; Shalu, 2016
Suspended growth systems		
Activated sludge	Mixing and addition of oxygen by aeration to convert organics into biomass. Removal of biomass by settling of flocs in clarifiers. Inorganic nutrients may be removed in biomass or by mineralisation.	Descoins *et al.*, 2012; Ahansazan *et al.*, 2014; Zheng *et al.*, 2016
Membrane bioreactor	Similar to the ASP, except that the solids are removed by filtration through membranes, decoupling reliance on floc-formation.	Baek and Pagilla, 2006
Oxidation ponds	Highly dependent on the environmental conditions. Treatment is achieved through the interaction of algae and bacteria. Bacteria digest and oxidize sewage constituents and render it harmless and odour free. Oxygen required to sustain bacteria is provided through photosynthesis which results from the use of carbon dioxide by algae. Solids settle down resulting in a clear effluent.	Butler *et al.*, 2017

2.2. The activated sludge process

The ASP is a suspended-growth aerobic system which has been widely adopted for treatment of domestic and industrial wastewater (Saunders *et al.*, 2016; Gonzalez-Martinez *et al.*, 2016; Jafarinejad, 2017). A conventional ASP (Figure 2.1) consists of two distinct unit operations performed in two separate and consecutive tanks namely, the aeration tank and the secondary clarifiers/sedimentation tank (Evans *et al.*, 2012; Attiogbe, 2013; Saleh *et al.*, 2014).

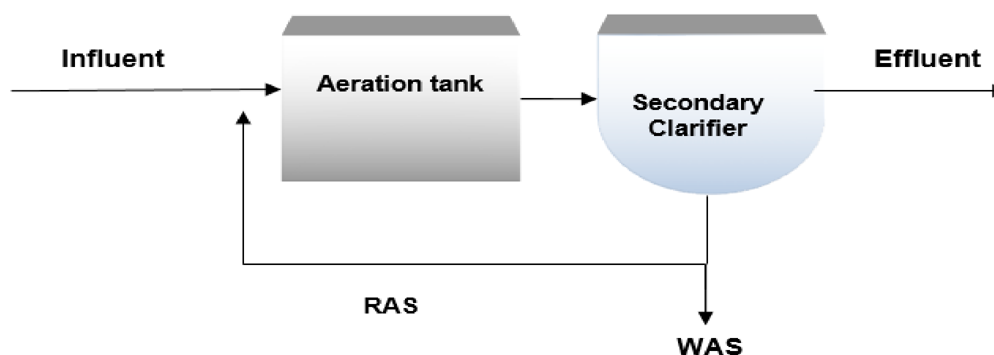


Figure 2.1: Basic configuration of conventional Activated Sludge Process. WAS=Waste Activated Sludge; RAS= Return Activated Sludge (adapted from Sastry *et al* ., 2013)

The ASP removes soluble and insoluble suspended organic matter from wastewater and converts this into a flocculent microbial suspension that settles in a subsequent gravity clarifier (Ramothokang *et al.*, 2003). This is primarily achieved through growth and maintenance of a large, diverse and active population of macro- and microorganisms (Xin-chun *et al.*, 2007; Hu *et al.*, 2013). The composition of the microbial population does not only depend on the influent wastewater, but also on the environmental parameters, as well as on operational conditions (Marrengane, 2007; Rustum, 2009).

In the ASP, the influent wastewater and RAS with 'activated' microorganisms is aerated and mixed. This suspension is referred to as mixed liquor or activated sludge (Evans *et al.*, 2012; Attiogbe, 2013). The resultant flocculent biomass that settles from the wastewater in a clarifier is referred to as a floc (Ramothokang *et al.*, 2003; Naidoo, 2005; Marrengane, 2007). In order to obtain sludge with good settling characteristics and subsequently good effluent quality, the growth of floc forming bacteria must be maintained (Lacko *et al.*,1999; Satoh *et al.*, 2013). Filamentous bacterial species play

a crucial role in floc-formation; they bind to floc-forming bacteria with biopolymers and provide a rigid support network or backbone to which floc-forming bacteria can adhere and proliferate (Milobeldzka *et al.*, 2016).

Aeration affects the dissolved oxygen (DO) level in mixed liquor, and is believed to have an effect on the growth ratio of filamentous to non-filamentous bacteria in the activated sludge (Wagner *et al.*, 2002; Martins *et al.*, 2004). The oxygen concentration is important in determining the efficiency of activated sludge settling. Low DO concentration can encourage the predominance of filamentous bacteria with the subsequent deterioration of effluent quality. On the other hand, high DO concentrations may result in poor settling characteristics and high concentration of solids in the effluent, air bubbles become entrapped in the flocs causing them to float. It is thus mandatory to keep DO at an acceptable level to ensure that biomass settles well, with subsequent good effluent quality (Martins *et al.*, 2004). To maintain DO, diffused or surface aeration systems, which also serve to maintain the mixed liquor in a completely mixed regime, are commonly used (Moral, 2004; Liu, 2012). The rate of degradation of organic matter is retarded if the DO is too low as a consequence of system overloading. Conversely, if the concentration is extremely low, the process becomes inefficient and is likely to waste energy while not effectively treating the effluent (Abu-danso, 2015).

On leaving the aeration tank (retention time typically 6 hours), the treated effluent flows into the secondary clarifier. The secondary clarifier is an important component of the system and performs two functions: sedimentation of wastewater through gravity, and thickening of sludge (Gray *et al.*, 2002; Carley, 2003). If floc formation and clarification are efficient, the clarified effluent will not contain suspended particles (Bai *et al.*, 2016). To determine the efficiency of the secondary clarifier, two parameters, mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS), are monitored by the WWTWs. They give an indication of the concentration of microorganisms (Abu-danso, 2015). If the concentration is excessively high, the process is susceptible to bulking.

To ensure process efficiency and continuity, a portion of the thickened activated sludge known as return activated sludge (RAS) from the secondary clarifier is continuously re-supplied into the aeration tank. Excess thickened sludge known as waste activated sludge (WAS) is removed from the system and is further treated and possibly reused (Spellman, 2003; Evans *et al.*, 2012; Ahansazan *et al.*, 2014). If the RAS rate is too low, solids will remain in the secondary clarifier thus resulting in loss of solids and septic return. If the RAS rate is too high, it may cause a hydraulic overload to the aeration

tank and reduced aeration efficiency, resulting in poor performance. Therefore, it is critical to keep the correct balance between RAS and WAS (Rustum, 2009). This is characterised by food to microorganism ratio (F/M). F/M is the amount of substrate available to the amounts of microorganisms in the reactor (Metcalf and Eddy, 2003). A low F/M may result in a bulking sludge, whilst a high F/M may cause dispersed growth due to excessive substrate that causes exponential growth (Moreno, 2004).

2.3. Enhanced Biological nutrient removal process

The enhanced biological nutrient removal (EBNR) ASP has become a recognised technology in wastewater treatment practice to control eutrophication and meet increasingly stringent effluent discharge standards (Hu *et al.*, 2013). A variety EBNR treatment process configurations exist. Certain systems are designed to reduce/remove Total Nitrogen (TN) while others reduce both TN and Total Phosphorus (TP) (Winkler *et al.*, 2011; Nourmohammadi *et al.*, 2013). Though the same configuration components of each system varies, BNR systems designed to remove TN must have an aerobic zone for nitrification and an anaerobic zone for de-nitrification, while BNR systems designed to treat TP must have an anaerobic zone free of DO and nitrates (Akpor, 2011).

2.3.1. Different zones in EBNR systems

2.3.1.1. Aerobic zone

The aerobic zone is a very important component of EBNR systems. It is a high DO concentration zone, where oxygen introduction is achieved by mechanical aeration (Zickefoose, 2004). During nitrification, ammonia is oxidised to the intermediate product nitrite by a group of autotrophs known as ammonium oxidising bacteria (AOB). Nitrite is then oxidised by nitrite oxidising bacteria (NOB) to nitrate (Schuler and Jenkins, 2003; Ramothokang *et al.*, 2006; Lochmatter *et al.*, 2014; Wang *et al.*, 2015). Nitrifiers are sensitive microorganisms, thus process parameters such as temperature, DO and pH must be monitored and adjusted to ensure efficient nitrification (Zickefoose, 2004)

In the case of biological phosphorus removal (BPR), heterotrophs known as phosphate accumulating organisms (PAO) takes up orthophosphate using energy derived from degradation of organic matter with nitrate or dissolved oxygen as the terminal electron acceptor and convert it to polyphosphate, which is then stored intracellulary (Blackall *et al.*, 2002; Mulkerrins *et al.*, 2004; Sathasivan, 2009). Phosphorus from bulk liquid phase is, therefore, removed through the removal of PAOs with the rest of the biomass

in the secondary clarifier (Baettens, 2001; Xing *et al.*, 2013; Zuthi *et al.*, 2013; Wang *et al.*, 2015; Chen *et al.*, 2016).

2.3.1.2. *Anaerobic zone*

This zone is free of both oxygen and nitrates. EBNR systems which operate with anaerobic zones are referred to as enhanced biological phosphorus removal (EBPR) systems. Under anaerobic conditions PAOs take up carbon sources such as volatile fatty acids (VFAs) from wastewater and store this in intracellular granules as PHAs. The energy required for this is provided by the hydrolysis of intracellular polyphosphate (poly-P) molecules (Hu *et al.*, 2003).

2.3.1.3. *Anoxic zone*

This zone is characterised by the presence of nitrates/nitrites, organic carbon and low or no DO. The anoxic zone is necessary for de-nitrification which is the second step in nitrogen removal (Zickefoose, 2004). De-nitrification is the removal of nitrate and excess nitrogen through conversion into nitrogen gas. This conversion is accomplished by several bacterial genera (Carrera *et al.*, 2003). In de-nitrifying systems certain critical parameters such as DO, temperature pH and organic carbon must be monitored to ensure stable de-nitrification (Zickefoose, 2004). De-nitrification is used when complete nitrogen removal from wastewater is required (Nourmohammadi *et al.*, 2013).

2.3.2. Wastewater treatment plant configurations

2.3.2.1. *Modified Ludzack-Ettinger (MLE) configuration*

MLE configuration (Figure 2.2A) is comprised of an anoxic zone followed by an aerobic zone. The anoxic zone is used for pre-denitrification where nitrates are recycled in two ways: they are returned in the RAS from the clarifier, and the internal recycle of mixed liquor directly from the aerobic zone (Lettie, 2006; Knapp, 2014). The typical retention times in the anoxic zone last between two and four hours (Ogunlaja, 2015). In the aerobic zone, enhanced organic utilisation takes place as well as nitrification of ammonia (Baettens, 2001).

2.1.1.1. *3-Stage Bardenpho configuration (3SB)*

The 3SB systems (Figure 2.2B) are designed for the removal of carbon, nitrogen, and phosphorus. This configuration incorporates the MLE technology. However, it is preceded by an anaerobic zone which provides appropriate conditions for PAO growth and uptake of volatile fatty acids or other carbon sources such as acetate to produce storage compounds such as PHA and PHB. Also polyphosphates are hydrolysed to

orthophosphates (Kendrick, 2011; Ogunlaja, 2015). Under subsequent aerobic conditions, PAOs use the stored PHAs as an energy source to take up soluble phosphorus, released under the anaerobic conditions, plus the phosphate originally present in the wastewater due to the difference in energetics between anaerobic and aerobic metabolism (Grady *et al.*, 2011). In the anoxic zone, the nitrate-rich mixed liquor is denitrified (Kendrick, 2011).

2.1.1.2. *University of Cape Town configuration*

The University of Cape Town (UCT) configuration (Figure 2.2C) uses a similar principle to the 3SB. The difference lies in that the UCT system has an additional internal mixed liquor recycle (MLR) step from the anoxic zone to the anaerobic zone. The RAS is returned to the anoxic zone rather than the anaerobic zone, which minimizes the effect of nitrate recycle to the anaerobic zone (Knapp, 2014; Vaiopoulou, 2014). The PAOs are therefore given a selective advantage of full access to all readily available biodegradable organic matter. The UCT process is used for the removal of both nitrogen and phosphorus (Ogunlaja, 2015). However, chances of adding nitrates from the aerobic-anoxic MLR still exist, which led to a modification of the UCT configuration. In the modified configuration, the anoxic zone is compartmentalised so as to further reduce the effect of nitrate recycle to the anaerobic zone (Baettens, 2001; Broch, 2008; Ogunlaja, 2015).

2.1.1.3. *5-Stage Bardenpho configuration*

The 5-stage Bardenpho (5SB) configuration (Figure 2.2D) is designed for both nitrogen and enhanced phosphorus removal (Baettens, 2001; Lettie, 2006). It is used where low concentrations of phosphorus and nitrogen are required in the final effluent, for example where the treated effluent will be discharged into environmentally sensitive areas. It incorporates the 3SB technology, with an additional anoxic zone and anaerobic zone for de-nitrification and nitrification and to ensure that no residual nitrates are present in the clarifier which can enter the RAS stream and retard the growth of PAOs (Motlagh and Goel, 2014).

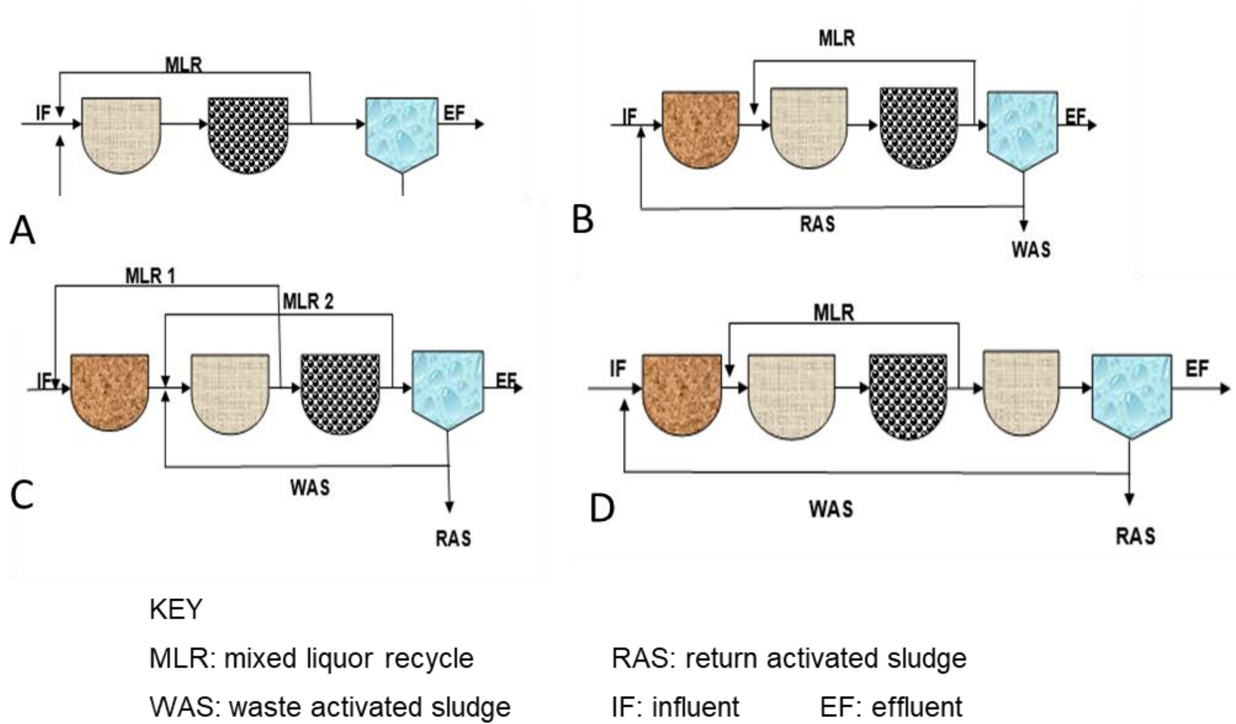


Figure 2.2: Schematic diagram representing activated sludge process configurations (A) MLE process, (B) Bardenpho process, (C) UCT process and (D) 5-stage Bardenpho process

Activated sludge separation problems

The ability of the ASP to produce an acceptable effluent with low suspended solid levels is reliant on the sedimentation process in the clarifier. Solid-liquid separation is considered to be a crucial step of wastewater treatment (Gerardi, 2002; Spellman, 2003; Hug, 2006; Hartley, 2008). However, there are several problems encountered with solid-liquid separation ever since the development of ASP (Table 2.3). Among these problems, those associated with the excessive growth of filamentous bacteria are filamentous bulking and foaming (Séka *et al.*, 2001; Ramothokang *et al.*, 2003; Schuler and Jassby, 2007).

Table 2.3: Solid separation problems classification (Adapted from Jenkins *et al.*, 2004)

Problem type	Problem description	Effect of the problem
Dispersed growth	Activated sludge comprises of numerous microorganisms existing as individual cells or small aggregates dispersed in the bulk liquid. The rate at which they sediment is too low for them to be removed by gravity sedimentation.	No settling of activated sludge, turbid effluent, and dilute RAS.
Pinpoint flocs	Activated sludge contains many poorly settling flocs. This normally results when there is an insufficient amount of exocellular material and/or filamentous bacteria for effective floc formation.	Low sludge volume index (SVI) and highly turbid effluent.
Rising sludge	Results from excess de-nitrification, which is caused by anoxic conditions in the aeration tank. Sludge particles attach to rising nitrogen bubbles and float on the surface of the secondary clarifier.	Activated sludge scum formation on the surface of the secondary clarifier. Turbid effluent.
Viscous (non-filamentous) bulking	Excess production of extracellular polymeric substances (EPS) by activated sludge bacteria causes activated sludge to be highly water retentive.	Decreased settling and compaction rate; virtually no solids separation in severe cases.
Filamentous bulking	Excessive growth of filamentous organisms interferes with compaction and settling of activated sludge. This common problem will be discussed in more detail in Section 2.4.1.	High SVI, unsettled sludge, low RAS.
Foaming/scum formation	Caused by (i) non-degradable surfactants and (ii) presence of *Nocardia* spp. and sometimes (iii) *Microthrix parvicella.* Discussed in Section 2.3.3.	A large amount of activated sludge solids and/or bacteria (foam) floats to the surface of the secondary clarifier and can overflow into the secondary effluent.

2.3.3. Filamentous bulking and foaming

Filamentous bulking is the most common and yet complex sludge separation problem encountered by ASP WWTWs globally (Juang, 2005; Mielczarek *et al.*, 2013; Liu *et al.*, 2016). It is a global concern since it results in the poor settle-ability of the MLSS and

subsequent poor effluent and sludge quality (Martins *et al.*, 2004; Wang *et al.*, 2015). Not only does sludge bulking result in heavy penalties due to nonconformity with discharge permits, but it also results in the need to apply expensive remedial methods (Zhao, 2012).

Two types of bulking problems exist, the first one is non-filamentous bulking which is due to excessive production of EPS by bacteria (Subramanian *et al.*, 2010; Mesquita *et al.*, 2011). This type of bulking sludge is rare and easily corrected by chlorination (Govoreanu, 2004; Bitton *et al.*, 2005). The most common and problematic is filamentous bulking caused by excessive growth of filamentous bacteria (Eikelboom, 2000; Schuler and Jassby, 2007; Rittmann and McCarty, 2012). Excessive growth of filamentous bacteria is in turn prompted by changes in process conditions (Naidoo, 2005).

In order to determine the sludge settling ability, SVI is used (Perez *et al.*, 2006; Mesquita *et al.*, 2008). SVI is defined as a measurement of sludge settle-ability after allowing mixed liquor to stand for 30 minutes in a 1L measuring cylinder (Richard, 2003; Wells, 2014). It is used in conjunction with the concentration of suspended solids. Studies have associated high SVI with an increase in filamentous bacteria abundance (Amaral and Ferreira, 2005; Hu *et al.*, 2013; Jassby *et al.*, 2014). Sludge with an SVI of ≤ 100 ml/g usually settles well in the clarifier (Mesquita *et al.*, 2008; Yousuf, 2013; Wells, 2014). A SVI ≥ 150 ml/g is often associated with bulking and foaming (Juang, 2005; Mesquita *et al.*, 2009).

Ideally, for good sludge settle-ability, there should be a balance between the growth of filamentous bacteria and floc-forming bacteria (Séka *et al.*, 2003; Marrengane, 2007). The most frequently observed filamentous bacteria in bulking sludge include: *M. parvicella*, *Sphaerotilus natans,* and Eikelboom Type 021N, Type 0041 and Type 0092 (Madoni *et al.*, 2000; Kanagawa, 2002). Depending on the type of filamentous bacteria present, interference can result in either open flocs or inter-floc bridging. The former occurs when copious filamentous bacteria grow inside flocs which are poorly consolidated, thus capturing water inside the flocs. The latter occurs when filamentous bacteria protrude from the flocs into bulk liquid, forming bridges between the flocs and preventing compaction of individual flocs (Richard, 2003; Liao *et al.*, 2004).

Foaming sludge is characterized by a considerable volume of foams and solids accumulating on the surface of aeration basins and settling tanks (Liu *et al.*, 2003; Khairnar *et al.*, 2014). There are two types of foaming that need to be clearly

distinguished from one another, namely, filamentous foaming caused by certain types of filamentous bacteria, and abiotic foaming caused by a high concentrations of surface active compounds such as oil and grease (Madoni *et al.*, 2000).

Biological foaming is a well-recognised ASP operational problem that results from the presence of certain filamentous microorganisms that have cell walls with hydrophobic properties similar to that of fats, oils, and grease. It is these hydrophobic properties that enable the bacterial bulk to float on the surface of the liquid in the bioreactor/s (Madoni *et al.*, 2000).

According to Lechevalier and Lechevalier (1974) and Pipes (2017) as cited by Rampersad (2002) and Wang *et al.* (2014), biological foaming is mainly caused by *Gordonia amarae*. However, recent studies have revealed that foaming is caused by a wider range of filamentous bacteria. These include other nocardioforms, *M. parvicella* and several Eikelboom morphological types (Rossetti *et al.*, 2005; Soddell *et al.*, 2006; Aonofriesei and Petrosanu, 2007; Wang *et al.*, 2016). Moreover, a number of research studies have shown filamentous bacteria to be associated with WWTWs in different regions (Wang *et al.*, 2014; Guo *et al.*, 2015), as well as WWTWs with different design, operational, and influent characteristics (Eikelboom, 2000; Khan *et al.*, 2013).

Several national surveys have been carried out in different countries including the United States (Mahamah, 2016), Italy (Madoni *et al.*, 2000), Australia (Seviour *et al.*, 1994), France (Pujol *et al.*, 1991), and South Africa (Blackbeard *et al.*, 1985, Lacko *et al.*, 1999, Welz *et al.*, 2014). All these studies aimed at gaining knowledge on the distribution of filamentous microorganisms in individual cities and/or countries as well as knowledge of proper control methods. The results lead to conclusions that growth of filamentous bacteria is induced by many different factors which include nutrient deficiency (Lou and Leong, 2015), DO concentration (Tay *et al.*, 2002; Martins *et al.*, 2004), substrate concentration gradient (Tsang *et al.*, 2008; Guo *et al.*, 2012), pH (Guo *et al.*, 2014), and temperature (Hu *et al.*, 2013). Different filamentous species are selected under different environmental conditions. Some important parameters associated with filament selection are given in Table 2.4 (Jenkins *et al.*, 2004; Deepnarain, 2014).

Table 2.4: Parameters associated with filamentous bacterial dominance (Adapted from Jenkins *et al.*, 2004)

Parameter	Filamentous microorganisms
Low F/M	*M. parvicella, Nocardia* spp., Eikelboom Type 0041, 0675, 0092, 0581, 0961, 0803, and *Haliscomenobacter hydrossis*
Low DO	Type1701, *Sphaerotilus natans,* 021N, *H. hydrossis,* and *Thiothrix* spp.
High sulphides	*Thiothrix* spp.*, Beggiatoa* spp.*,* Type 021N, and Type 0914
Nutrient (nitrogen, phosphorus) deficiency	*Thiothrix* spp.*, H. hydrossis,* Type021N, and *S. natans,*

2.3.4. Bulking and foaming incidences in South Africa

Blackbeard *et al*. (1985) conducted a study on 33 BNR WWTWs in South Africa and found that filamentous bulking was common in South African WWTWs at that time. The most frequently dominating filamentous bacteria isolated from mixed liquor samples during this study in descending order were found to be: Type 0092, Type 0675, Type 0041, *M. parvicella*, Type 0914, and Type 1851. Type 0092 and *M. parvicella* were frequently observed in low F/M ratio conditions. Type 0092, *M. parvicella*, Type 0041, and Type 0675 and 0914 were most frequently observed in foam samples, with Type 0092 having the highest incidence (Blackbeard *et al*., 1985).

However, a later study by Lacko *et al.* (1989), with the objective of identifying filamentous bacteria present in ASP plants in Durban and surrounding areas (South Africa), identified a diverse filamentous bacteria population. Dominant filamentous bacteria identified in descending order were: *Nocardia* spp., Type 0041, Type 0675, Type 1851, Type 021N, *Nosticola limicola*, *S. natans*, *Thiothrix* I and II. *Beggiatoa* and *Nocardia* spp. were the only dominant filamentous bacteria present in foam samples, while Type 0914, *M. parvicella* and *S. natans* occurred incidentally. From this study, it was mentioned that all filamentous bacteria were present throughout the year, while *Nocardia* spp*. and M. parvicella* were dominant during the winter months. It was, therefore, concluded that filamentous bacteria are significantly affected by seasonal and influent variations.

A more recent study by Welz *et al.* (2014) compared filamentous populations in AS from 11 WWTWs in the Cape Town area (South Africa) with previous studies. In descending order, the most dominant filamentous bacteria were found to be Eikelboom Type 0092, Eikelboom Type 1851, Nocardioforms, *M. parvicella*, and Eikelboom Type 021N.

From all three studies, there were some noticeable differences. The most important being the fact that Eikelboom Type 0092 was present in almost all samples from the first and the last study; however, it was not identified in any samples from the second study. Eikelboom Type 0092 is well documented as a dominant filament in ASPs across the world, including Europe and Australia. However, its absence has been documented in other locations (Speirs *et al.*, 2009). Thus, it can be suggested that dominant filamentous bacteria vary from region to region depending on the type of influent as their growth depends on different biochemical and physiological needs, environmental conditions and operating parameters (Martins *et al.*, 2004).

2.4. Methods used to control filamentous bulking and foaming

Control of filamentous bulking and foaming remains a challenge facing the field of wastewater treatment globally (Janczukowicz *et al.*, 2001; Ramothokang *et al.*, 2003; Hug, 2006; Speirs *et al.*, 2009; Abusam *et al.*, 2016). To suppress overgrowth of filamentous bacteria, two approaches are followed, namely: specific and non-specific methods (Khairnar *et al.*, 2014; Table 2.5). Specific methods are aimed at removing the cause of filamentous proliferation and are targeted to a specific microorganism or group of microorganisms. Non-specific methods are aimed at reducing the levels of filamentous bacteria without any effect on the cause of their growth (Tandoi *et al.*, 2006), thus only providing a temporary solution (Mamais *et al.*, 2011).

Table 2.5: Methods used to control filamentous bulking and foaming

Non-specific methods	
Chlorination	Chlorine is a popular and well documented non-specific method used to control the overgrowth of filamentous bacteria (Saayman, 1999; Jenkins *et al.*, 2004; Caravelli *et al.*, 2006; Salem *et al.*, 2014). The principle behind the addition of chlorine to bulking sludge is that it should kill exposed outer filamentous bacteria while floc formers remain viable within the floc (Ramirez *et al.*, 2000; Richard, 2003). However, it is non-selective and may therefore hinder both nitrification and biodegradation of organic matter, resulting in poor effluent quality (Séka *et al.*, 2003). Many successful cases have been reported where chlorine has been used in accordance with recommendations (Caravelli *et al.*, 2006; Zuthi *et al.*, 2013; Saleh *et al.*, 2014). However, unsuccessful cases have also been reported. A study by Guo *et al.* (2012) showed Eikelboom Type 021N to be resistant to chlorination. Another negative effect of using this biocide as a control measure is that it produces undesirable by-products such as trihalomethanes which can be dangerous to human health (Richard, 2003; Caravelli *et al.*, 2006).
Hydrogen peroxide	Hydrogen peroxide (H_2O_2) has been used for bulking control in a similar way to chlorine. H_2O_2 destroys filamentous micro-organisms by degrading their sheaths. Regardless of the mechanisms, the effect is similar to that of chlorine is used (Schuler and Jenkins, 2003). Since H_2O_2 dissociates to water and oxygen, it does not leave any toxic residuals thus making it advantageous over chlorine (Wells, 2014). Although its dose and application time for effective filament reduction and bulking control vary from plant to plant, they are generally higher than for chlorine (Hammadi *et al.*, 2012), therefore, making H_2O_2 expensive (Govoreanu, 2004).
Synthetic polymer addition	Synthetic polymer addition is another method used to control bulking sludge. Here, the sludge is compacted resulting in increasing the settling rate and inhibiting filamentous bacterial growth. However, it offers a temporary solution. A study by Juang (2005) showed that the addition of synthetic polymer solved poor sludge settle-ability temporarily and when polymer addition was stopped, sludge bulking reappeared and was more severe than before. It was then showed that the addition of synthetic polymer causes a shift in the microbial population and affects the growth of floc-formers in activated sludge. Thus, it was concluded that synthetic polymer addition is an unsuitable alternative to control poor settling sludge (Juang, 2005; Juang and Chiou, 2007).

Table 2.6: ***Cont.*****:**

Specific methods	
Biological selectors	Biological selectors can be defined as mixing basins or channels (Martins *et al.,* 2004; Liao *et al.,* 2006). They suppress the growth of filamentous bacteria by providing a high substrate environment in the selector which favours proliferation of floc-forming bacteria (Martins *et al*., 2004; Gray *et al*., 2006). Successful cases on the use of biological selectors to control both filamentous bulking and foaming have been reported (Azimi and Zamanzedeh, 2006; Khairnar *et al*., 2014). Although selectors are relatively cost effective in terms of construction and operation, they have limitations. They can be too large or too small in size to properly function (Gray *et al*., 2006).
Bacteriophage-mediated control	Bio-control of filamentous bulking and foaming is an unexplored technology in AS treatment plants (Kang, 2013). Bacteriophages are bacterial viruses that are highly specific in their host-cell recognition. They are found in all habitats where their host bacteria proliferate (Clokie *et al*., 2011; Mamais *et al*., 2011; Phumkhachorn, 2012). They have been isolated from surface water, soil, AS and sewage (Jończyk *et al*., 2011; Kumari *et al*., 2011). Phage-based therapy for infectious disease treatment has been extensively investigated, with promising results (Kumari *et al*., 2011; Jassim and Limoges, 2014). Along similar lines, it is possible that bacteriophages may be exploited as bio-control agents for filamentous bacteria implicated in sludge bulking and foaming.

2.5. Identification of filamentous bacteria

Engineers and microbiologists are constantly looking for ways to improve system design and performance by controlling or preventing filamentous bulking and foaming (Naidoo, 2005). The understanding and characterisation of bulking sludge is generally thought to be based on proper identification of the filamentous bacteria in question (Richard, 2003; Martins *et al*., 2004).

2.5.1. Isolation of filamentous bacteria

Isolation of filamentous bacteria using culture-based methods is a promising approach for investigating factors that promote or inhibit their growth (Kämpfer, 1997) as cited by (Ramothokang *et al.,* 2003). However, it is still regarded as a daunting task. In most cases, attempts to isolate filaments are found to be largely unsuccessful (Khan *et al*., 2013). This is because in a laboratory setting, the majority of slow growing filamentous bacteria are outgrown by rapid floc-formers (Ramothokang *et al*., 2003).

To eradicate this, various procedures have been developed. Van Veen (1973) as cited by Kämpfer (1997) developed an isolation method based on specific dilution procedures. Ziegler *et al.* (1990) reported that sonication prior to plating plays a crucial role in separating floc-formers from filamentous bacteria and reducing their numbers. Micro-manipulation with special micro tools under a microscope is another method used, however, it is very expensive and thus inaccessible to many laboratories (Ramothokang *et al.*, 2003).

2.5.2. Phenotypic identification

Identification of filamentous bacteria based on phenotypic characteristics such as morphology and staining characteristics has been widely used (Eikelboom, 2000; Oerther *et al.*, 2001; Rossetti *et al.*, 2005; Seviour and Nielsen, 2010). However, this approach has its limitations (Oerther *et al.*, 2001). Microscopic identification of filamentous bacteria based on morphology requires well-trained personnel otherwise an incorrect result may be obtained (Martins *et al.*, 2004).

The morphology is strongly dependent on environmental factors (Ramothokang *et al.*, 2006). It has been found that the morphology and staining reaction of many filamentous bacteria can vary in different environmental conditions (Alonso *et al.*, 2002; Marrengane, 2007). Non-filamentous forms of *S. natans*, *N. limicola*, Eikelboom Type 1863 and *Nocardia* spp*.,* amongst others, have been documented (Ziegler *et al.*, 1990; Ramothokang *et al.*, 2006; Marrengane, 2007; Faheem, 2013; Seder-Colomina *et al.*, 2015). Furthermore, some filamentous bacteria such as *M. parvicella* have Gram variable stain reactions (Kämpfer, 1997).

Although microscopic characterisation has its limitations, it is valuable for presumptive identification and determining the physical nature of the activated sludge flocs (Jin *et al.*, 2011). Due to the aforementioned reasons, a holistic approach using both conventional (phenotypic) and molecular (phylogenetic) identification methods is necessary for accurate identification of filamentous bacteria (Marrengane, 2007).

2.5.3. Molecular identification

Molecular techniques based on molecular markers such as ribosomal RNA (rRNA) proved to strongly support identification and enumeration of activated sludge communities (Piterina *et al.*, 2010). The most commonly used phylogenetic marker is the 16S rRNA gene (Lane *et al.*, 1985; Martins *et al.*, 2004; Hugenholtz, 2002). The

rRNAs are targeted because they are present in all living cells in high copy numbers (Amann *et al.*, 1997; Srinivasan *et al.*, 2014). The genes encoding for the rRNAs are highly conserved across phylogenetic domains, with some regions more conserved than others (Schramm *et al.*, 1999). The 16S rRNA approach consists of DNA extraction, determining DNA integrity and concentration, and subsequent PCR amplification of the target gene followed by sequencing (Suzuki and Giovonnoni, 1996).

2.5.3.1. *Polymerase Chain Reaction*

It is widely accepted that only a small fraction of the bacterial community has been isolated in pure cultures, especially from marine ecosystems (Kaeberlein *et al.*, 2002; Vartoukian *et al.*, 2010; Stewart, 2012) and activated sludge (Saikaly *et al.*, 2005). However, with the advent of small-subunit rRNA-based molecular fingerprinting techniques such as PCR it has become possible for environmental engineers and scientists to assess bacterial diversity in activated sludge systems more accurately (Saikaly *et al.*, 2005).

PCR is one of the most widely used culture-independent techniques in the analysis of activated sludge communities and their functions (Amann *et al.*, 1995; Clarridge, 2004). The 16S rRNA gene has been targeted to determine the overall population biodiversity. This gene is approximately 1 500 bp in size (Kimura *et al.*, 2006). Primers targeting the 16S rRNA have been widely used to obtain amplicons directly from the DNA which has been extracted from activated sludge (Rosselli *et al.*, 2016). Universal and domain targeted primers for the 16S rRNA gene are still widely used (Sambo *et al.*, 2018). For a successful PCR amplification, primer specificity is crucial, as when amplifying a desired sequence, a primer should be complimentary to the target sequence only (Wang and Seed, 2003).

2.5.3.2. *Next Generation Sequencing*

It is common knowledge and widely accepted that only small fractions of bacterial communities have been isolated in pure cultures. Thus, a variety of culture-independent methods have been developed to carry out comparative analysis of microbial communities and to relate community composition to environmental parameters (Ramette, 2009; Zapka *et al.*, 2017). Such methods include Terminal Restriction Fragment Length Polymorphism (T-RFLP) and amplicon sequencing, including Next Generation Sequencing (NGS) (Lindström *et al.*, 2018).

Recent developments in high-throughput DNA sequencing techniques made NGS methods the most attractive alternatives for microbial community analysis (Lindström

et al., 2018). In contrast to T-RFLP, NGS methods reveal taxonomic identity to the extent that designated sequence data is available (Prakash *et al.*, 2014). However, generating NGS datasets can be very costly, and require more complex bioinformatics interpretation. In addition, the protocols and methods used for microbial NGS analysis are not well standardised yet (Kulkarni and Frommolt, 2017).

The next chapter gives a descriptive information of material and methods utilized to isolate and identify filamentous bacteria, their co-occurring bacteriophages and to determine their abundance in wastewater ASPs.

CHAPTER 3
METHODOLOGY

3.1. Section 1: Isolation of filamentous bacteria and bacteriophages

This section of the chapter presents materials and methods for the isolation of bacteria and their co-occurring bacteriophages from WWTWs in the Cape Town area. The purpose of this was to obtain a representation of the bacterial population implicated in bulking and foaming sludge in the Cape Town area and to explore the potential of bacteriophages in controlling over-proliferation of filamentous bacteria in activated sludge (AS).

3.1.1. Wastewater treatment plants (WWTWs)

Six different AS WWTPs in the Cape Town area were investigated for the presence of filamentous bacteria and their co-occurring bacteriophages. Of these, two were operated in the 5-stage Bardenpho (5SB) configuration, two operated in the Modified Ludzack-Ettinger (MLE) configuration, one operated in the 3-stage Bardenpho (3SB) configuration, and one in the University of Cape Town (UCT) configuration. For the purpose of this study, these plants were designated as 5SB_A, 5SB_B, MLE_1, MLE_2 and 3SB_C and UCT, respectively. Surface aeration is utilised in 5SB2, MLE1 and UCT, diffuse aeration is applied in 5SB_A, and both surface and diffuse aeration systems are utilised in MLE_B. Permission was obtained from the wastewater treatment plants to sample all sites, and this study received ethics clearance from the Cape Peninsula University of Technology.

3.1.2. Sampling

Samples were collected from all six WWTWs over a period of six months. Sample collection was performed once every month from November 2016 to May 2017. Grab samples of mixed liquor and foam were taken from the aerobic zones of each plant and stored in half filled autoclave pre-sterilised sample bottles to maintain aerobic conditions for the survival of filamentous bacteria during transit (Eikelboom, 2001). Both mixed liquor and foam samples were kept on ice in cooler boxes during transit and were analysed immediately in order to reduce any changes in their characteristics.

3.1.3. Sludge pre-screening

Prior to isolation, the sludge volume index (SVI) of all the samples was determined according to the standard method. Briefly, 100 ml of mixed liquor sample was

transferred into a 100 ml measuring cylinder and allowed to settle for 30 minutes as shown in Figure 3.1. The volume of the settled sludge was read from the interface of the sludge water. For accurate results, foaming and bubbles were avoided by pouring gently. SVI was determined mathematically from Equation 1. All samples with the SVI above 180 ml/g were used to isolate filamentous bacteria and those with SVI below 180 ml/g were discarded.

$$SVI = \frac{SV}{MLSS} x100 \qquad \text{Equation 1}$$

SV = Sludge volume after 30 minutes
MLSS = Mixed liquor suspended solids concentration

Figure 3.1: Photograph of the sludge volume index (SVI) test being conducted in measuring cylinders before and after settling of the activated sludge.

3.1.4. Bacterial isolation and characterisation

All samples were centrifuged at 1 500 rpm for 10 minutes to separate floc-forming bacteria from filamentous bacteria via gradient separation. The samples were then serially diluted (10^{-1}-10^{-5}) in sterile distilled water, homogenised using a vortex mixer for 10 seconds and sample aliquots of 0.1 ml from the dilutions were dispensed onto various agar media (Table 3.1 and appendix 1) as described by Ramothokang *et al.* (2003). Spread plate and streak plate techniques were used throughout the isolation

procedure. The plates were incubated at 37°C for 1 week. After incubation, well defined and morphologically unique colonies were selected, purified by streak plating onto selected agar plates and incubated at 37°C for 3 to 5 days. To determine the morphological characteristics, all isolates were Gram stained (appendix 2) and observed using a light microscope with an oil immersion lens under 1 000x magnification. The purified cultures were stored in 10% (v/v) glycerol stock at -80°C until further use.

Table 3.1: Media used for isolation of filamentous bacteria (adapted from Ramothokang *et al.*, 2003)

Media	Filamentousbacteria reported to grow
R2A	*M. parvicella*, *S. natans*, Eikelboom Types 1701, 0803, 1863, 0092, 0411, and *Leptothrix* spp. (Kamper *et al.*, 1997)
Casitone glycerol yeast agar (CGYA)	*S. natans* (Kamper *et al.*, 1997)
Glucose yeast extract agar (GYA)	*Nocardia* spp. (Davenport, 2008)
Tryptone yeast glucose agar (TYGA)	*Nocardia* spp. (Kamper *et al.*, 1997)

3.1.5. Bacteriophage isolation

Potential bacteriophages were isolated from wastewater samples as per the method described by Khairnar (2014). Briefly, 20 ml of mixed liquor sample was centrifuged at 8 000 rpm for 20 minutes and filter sterilised with a 0.45 µm membrane filter to remove the cells and debris. For bacteriophage enrichment, 1 ml of the filtrate was inoculated with 1 ml of filamentous bacteria overnight culture. The contents were left at room temperature for 1 hour without shaking to encourage bacteriophage adsorption before further incubation with shaking (90 rpm) at 37°C for 48 hours.

After enrichment, 3 ml of chloroform (10% v/v) was added to lyse phage-infected bacterial cells, releasing the bacteriophages. Thereafter, the samples were vigorously shaken for 30 minutes, followed by centrifugation at 8 000 rpm for 15 minutes to collect the supernatant. The contents were then filter-sterilised using a 0.45 µm membrane filter. The centrifugation and filtration steps were carried out to remove bacterial cell debris from the samples. The filtrate was used as an enriched lysate for isolation of phages using the spot test method as described by Petrovski *et al.* (2011) with some modifications.

The presence of bacteriophages was tested using a spot test method described by Petrovski *et al.* (2011) with some modifications. Briefly, 100 µl of host bacteria was spread on a sterile GYEA plate, allowed to dry and incubated at 37°C for 2 hours. After incubation, 20 µl lysate was spotted on the plate and incubated at 37°C for 3 to 5 days for detection of lytic spots over the bacterial lawn on the agar plate.

Molecular identification

3.1.6. Starter culture preparation

To prepare working cultures, each isolate was grown on their respective solid media (Table 3.1) at 37°C. These were then introduced into 10 ml liquid medium and grown in an orbital shaker at 110 rpm at 37°C for 48 hours and used for DNA extraction.

3.1.7. DNA extraction

DNA was extracted using the phosphate, sodium dodecyl sulfate (SDS), chloroform - bead beater (PSC-B) method modified for DNA extraction from *Actinobacteria* (Appendix 3). The method is based on the procedure described by Miller *et al.* (1999). Briefly, cultures were centrifuged to collect cell pellets, followed by washing in phosphate buffer and re-centrifugation at maximum speed for 5 min. The resultant pellets were re-suspended in 300 µl phosphate buffer and added to bead-beater vials. 300 µl SDS lysis buffer and 300 µl chloroform:isoamyl alcohol were added to the cell bead-beater vial and incubated at room temperature for 15 minutes. The contents were vortexed then centrifuged to pellet the cell debris. The supernatants were transferred to sterile microfuge tubes to which 7M ammonium acetate was added to achieve a final concentration of 2.5M. The contents were mixed by hand and centrifuged. The supernatants were transferred into sterile tubes, to which 315 µl isopropanol was added and the tubes were incubated for 2 hours. After incubation, these were centrifuged, the supernatants carefully removed, and the pellets washed with 70% (v/v) ethanol. Thereafter, the ethanol was removed and the pellets were allowed to air dry. Once the trace ethanol had evaporated, the pellets were re-suspended in 10 mM Tris-HCl (pH8) and stored at 4°C for further use.

3.1.8. Agarose gel electrophoresis

Subsequent to DNA extraction, 1 µl of DNA sample was mixed with 2 µl loading dye (Thermo Fisher Scientific) and electrophoresed in a 1.5% (w/v) agarose gel (Appendix 4), alongside a 50 bp molecular weight marker (Thermo Fisher Scientific), at 80 volts for 60 minutes. The gel was viewed using Biorad Gel-doc imaging system (Hercules,

USA). This was performed to determine the integrity and concentration of the extracted DNA.

3.1.9. Polymerase Chain Reaction (PCR)

The amplification of the 16S rRNA gene was performed in total PCR reaction volumes of 25 µl using universal primer pairs F1: 5'- AGA GTT TGA TCI TGG CTC AG -3' and R5: 5'– AGC GIT ACC TTG TTA CGA CTT -3' (Cook and Meyers 2003). Each reaction consisted of: 12.5µl DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific), 1 µl each of 10 µM forward and reverse primers, 2 µl of genomic DNA and made to a final volume of 25 µl with nuclease free water. The PCR amplification was carried out using a Techne Touchgene Gradient PCR Thermal cycler under the following conditions: initial 2 min denaturation at 96°C; 30 cycles of: 45 sec denaturation at 96°C, 30 sec annealing at 55°C and 2 min extension at 72°C; followed by a final 5 min extension at 72°C and holding at 10°C. The PCR products were visualised using a 1.5% (w/v) agarose gel as indicated in Section 3.1.8.

3.1.10. Phylogenetic analysis

Sequencing of the PCR amplicons was performed by a commercial service provider (Inqaba Biotech, South Africa) using the Sanger method by BigDye® Terminator v3.1 Cycle Sequencing on ABI3500XL sequencer. Visualisation and preliminary analysis of sequences was performed using Chromas Version 2.6.6 (Technelysium Pty Ltd). Edited sequences were assembled using DNAMAN version 4.13 (Lynnon BioSoft). EzBioCloud available at: https://www.ezbiocloud.net/resources/16s_download (Accessed: 29 October 2018) was used to confirm the identity of the organisms.

3.2. Section 2: Next generation sequencing

This section is an addition to a microscopy study that was previously carried out to identify dominant filamentous bacteria in AS (Welz et al., 2018). Fifty samples with most dominant filamentous bacteria were selected for next generation sequencing (NGS) and qPCR analysis to confirm the previously identified filamentous bacteria and to further determine if there were any discrepancies between the two methods used. The fifty samples used were taken from six plants, three of which were in Cape Town (CPT) and the other three from Gauteng (GAU). CPT plants were designated C_MLE, C_UCT and C_3SB, GAU plants were G_MLE, G_3SB and G_3SB_B.

3.2.1. Quantitative polymerase chain reaction

DNA quantity and quality was determined using a Thermo Fisher Scientific (Waltham, USA) NanoDrop2000 instrument. The quantitative PCR (qPCR) reactions were performed using the primer pair 515F-Y (Parada *et al.*, 2016) and revised 806-R (Apprill *et al.*, 2015) in triplicate using 1 µl of template DNA with 2X Universal Taqman (Applied Biosystems, Foster city, USA) PCR Mastermix in a StepOnePlus (Applied Biosystems) Real-Time PCR System according to the manufacturer's instructions. The PCR reaction was carried out with an initial holding stage of 50°C for 2 minutes followed by 95°C for 10 minutes. The cycling stage consisted of 40 cycles of 95°C for 15 sec followed by 60°C for 1 min. DNA from *Escherichia coli* was used as linear standard to estimate the concentrations of bacterial cells in the samples.

3.2.2. Sequencing

NGS was performed using the Illumina (San Diego, USA) MiSeq workflow as per the manufacturer's guidelines at MR DNA (Shallowater, USA). Pooled equimolar amounts of metagenomic DNA from duplicate extractions from each AS sample were used to amplify the V4 region of the 16S rRNA gene using the primer pair 515F-Y (Parada *et al.*, 2016) and revised 806-R (Apprill *et al.*, 2015), with the forward primer being barcoded. An initial denaturation step at 94°C for 3 min was followed by 30 cycles of denaturation (94°C for 3 min), annealing (43°C for 40 sec), and extension (72°C for 1 min) using the HotStarTaq Plus Master Mix kit (Qiagen, Hilden, Germany). The PCR products were quality checked by visualisation in 1% (w/v) agarose gel. Aliquots of samples containing equimolar amounts of DNA were pooled and purified using calibrated Ampure XP beads (Beckman Coulter, Brea, USA) and used to prepare the DNA library according to the Illumina TruSeq protocol. Amplicon sequencing was performed using an Illumina MiSeq instrument according to the manufacturer's instructions. The data was analysed using the MR DNA analysis pipeline: The sequences were joined, and the barcodes were removed. Then, the sequences with <150 bp and/or ambiguous base calls were removed, and the sequences were denoised. Operational taxonomic units (OTUs) were then generated, and chimeras were removed using UCHIME (Edgar *et al.*, 2011). The OTUs were defined by clustering at 3% divergence using UCLUST (Edgar, 2010). The OTUs were assigned taxonomic classification using BLASTn against a curated database derived from the Ribosomal Database Project II (RDP II) and the National Centre for Biotechnology Information (NCBI) databases (http://rdp.cme.mus.edu and

http://www.ncbi.nlm.nih.gov). Filamentous bacteria obtained in this study were identified through Microbial Database for Activated Sludge (MIDAs), a curated database for organisms in activated wastewater treatment systems. All species obtained in this study were compared against those listed on Microbial Database for Activated Sludge 3 (MIDAS) 3 reference database and were further classified into their phylum groups.

3.2.3. Estimated absolute abundance

The formula below was used to calculate estimated absolute abundance of the filamentous bacteria obtained in this study. The product of a comparable relative abundance multiplied by the total bacterial count yields estimated absolute abundance. (Zhang *et al.*, 2017). In this study, the relative abundance was obtained from the 16S rRNA MiSeq analysis, while the estimated absolute microbial cell numbers were quantified by qPCR.

$$Relative\ abundance\ of\ a\ taxon\ \times Total\ bacterial\ counts = Estimated\ absolute\ abundace$$

…………..…..Equation 2

3.3. Data analysis

The NGS data obtained was analysed using PRIMER 7 software, the data was square-root transformed then subjected to statistical analysis to determine the microbial community composition similarity and diversity of the analysed samples. Resemblance patterns among samples were observed graphically by the ordination method, nonmetric multidimensional scaling (nMDS) based on similarity matrices calculated with Bray-Curtis similarity index. Analysis of variance (ANOVA) was used to determine the significance of similarity and diversity. Estimated absolute abundance of filamentous bacteria was presented as averages in plots constructed using Microsoft Excel. Furthermore, the significance of analysed variables on abundance of filamentous bacteria were analysed using analysis of variance (ANOVA).

CHAPTER 4
Isolation and identification of filamentous bacteria and bacteriophages: Results and Discussion

4.1. Isolation and identification of filamentous bacteria from activated sludge

The results obtained from attempts to isolate filamentous species from the City of Cape Town wastewater treatment plants are described and discussed in this Section. The species were isolated from monthly samples taken over a period of six months (November 2016 to May 2017). Sludge volume index (SVI) values for the sampled AS systems were generally greater than 150 ml/g during the course of this study. Microscope examination of AS and foam samples prior to culturing revealed the presence of bacteria which had filamentous morphology (data not shown).

Filamentous bacteria isolation from mixed liquor and foam samples by direct plating was largely unsuccessful. However, dilution and centrifugation of samples prior to plating on solid media facilitated the isolation of some filamentous-like bacteria. Twelve bacterial isolates with filamentous-like morphology were isolated from AS and foam samples, their DNA was extracted and identified through sequencing.

All the isolates formed colonies within 3 to 5 days of incubation at 37°C. The Gram-staining and morphological characteristics of the isolates are summarised in Table 4.1. To verify the Gram results and purity of the isolates, microscope-based examination was performed every fortnight. Initially, all isolates displayed a filamentous morphology. However, morphological shifts were observed from filamentous form to single cells upon sub-culture.

High quality DNA was extracted from 8 of the 12 original isolates and was identified by sequencing of the 16S rRNA gene. DNA sequencing revealed that 60% of the isolates from this study were affiliated to the *Gammaproteobacteria* class while the remaining 40% were affiliated to the Class *Bacillus*. Culture 5 and 11 initially displayed filamentous forms. However, after sub-culturing, a morphological shift was observed. Culture 5 was isolated as a Gram-positive, long-coiled filament but later appeared as single rods that were slightly curved (Figure 4.1A and Figure 4.1A_1). Culture 11 was also isolated as a Gram-positive, long-coiled filamentous bacteria, which later shifted to short filamentous bacteria. The 16S rRNA sequencing of culture 5 and culture 11 revealed that both these cultures were significantly similar to *Bacillus siamensis* by 99.86%, suggesting that these two isolates are closely related. *Bacillus siamensis* is a Gram-positive member of the genus *Bacillus. Bacillus* cells are rod-shaped and may occur singly, in short

chains and as filamentous bacteria (Logan and De Vos, 2015). They are commonly found in AS systems (Mizuki *et al.,* 2001; Hatamotoa *et al.*, 2017). Trick *et al.* (1984) was the first to isolate filamentous *Bacillus* species from bulking sludge. Ajithkumar *et al.* (2001) also isolated a filamentous bacterium which was a member of the genus *Bacillus* with no close relative at species level. Jorgensen *et al.* (1997) isolated a filamentous *Bacillus cereus*. However, all these are not associated with the Eikelboom type filamentous group, no Eikelboom type *Bacillus* filament is listed on MIDAS.

Also showing close similarity to the Class *Bacillus*, was culture 17. This isolate was initially isolated as a short, curved filament and maintained this filamentous form even after sub-culturing. The 16S rRNA gene sequencing of culture 17 revealed a significant similarity of 99.9% to the 16S rRNA gene sequence of *Enterococcus faecalis. Enterococcus faecalis* is a Gram-positive bacterium usually in coccoid form. *Enterococcus* species are part of the natural intestinal flora of humans and animals, and thus are widely distributed in wastewater (Sanderson *et al.*, 2019). Filamentous forms of *Enterococcus faecalis* have not been described, nor isolated from AS. Although sterile procedures were followed during sub-culturing, and this culture appeared to be pure when viewed under the microscope, there is always a possibility of contamination. Their persistence in wastewater makes *Enterococcus* species a suitable indicator of faecal contamination (Boehem and Sassoubre, 2014).

Culture 1 was isolated as a long-coiled Gram-positive filament which later shifted to short curved filamentous bacteria after sub-culturing. Culture 1 could not be sequenced due to low DNA yield.

Enterobacter ludwigii is the member of the *Enterobacter cloacae* complex which is of clinical significance accounting for 7% of nosocomial infections in hospital settings (Li *et al.*, 2015). According to literature, members of the *Enterobacter* genus are usually bacilli (Morand *et al.*, 2009; Davin-Regli, 2015; Annavajhala *et al.*, 2019; Morales-López *et al.*, 2019). In this study, culture 7 and 21 which revealed a significant 16S rRNA gene sequence similarity of 99.7% and 99.8%, respectively, to the 16S rRNA gene sequence of *Enterobacter ludwigii* displayed filamentous-like forms during isolation. Culture 7 was isolated as filamentous-like and maintained this morphology even after sub-culturing as shown in Figure 4B and Figure $4B_1$. The same was observed for culture 21.

Other members of this class were two Gram-negative isolates (culture 19 and culture 20). Culture 19 was isolated as a filamentous-like bacterium, which shifted to rod forms after sub-culturing (Figure 4.1C and Figure $4.1C_1$ respectively). The same was observed for culture 20. Their 16S rRNA gene sequencing revealed that these cultures have 99.5% and 99.6% sequence similarity, respectively, to *Serratia marcescens*

subsp. *marcescens*. *Serratia marcescens* subsp. *marcescens* is also known as an important cause of nosocomial infection (Mahlen, 2011). In wastewater, this bacterium was first isolated by Ajithkumar *et al*. (2003) from a domestic wastewater plant. Although there have been no reports on *Enterobacteriaceae* filamentous bacteria from wastewater, according to literature *Enterobacteriaceae* isolates produce filamentous bacteria in response to beta-lactam antibiotics (Buijs *et al*., 2008; Gould et al., 1997). Kuo *et al*. (2013) also isolated filamentous *Serratia* species from paper machine biofilm.

Culture 18 is a Gram-negative bacterium which was initially isolated as filamentous-like and later shifted to rod shaped cells. The 16S rRNA gene sequence of culture 18 was shown to be 99.8% similar to the 16S rRNA gene sequence of *Klebsiella variicola. Klebsiella variicola* is an emerging human pathogen responsible for infections such as respiratory tract infections (Martin and Bachman, 2018). This bacterium can be found in several environments such as rivers and wastewater (Rodríguez-Medina *et al*., 2019). *K. variicola* is considered to be a useful industrial strain, and has been used in many biotechnological applications, including wastewater treatment (Rodríguez-Medina *et al*., 2019).

Four out of nine isolates exhibited cellular morphology that is typical of filamentous bacteria throughout the study; while the remaining isolates shifted from the filamentous to single cell form upon repeated sub-culturing. A morphological shift has been recorded in literature where Eikelboom Type 1863 was observed as cocci-bacilli in pairs and sometimes as single cells (Saviour *et al*., 1997; Kampfer and Wagner 2002; Khan *et al*., 2012). Microscope-based identification of filamentous bacteria is based on characteristics such as morphology, Gram-staining, presence or absence of cell inclusions and biochemical fingerprint (Rossetti *et al*., 2006). However, it is generally accepted that morphology strongly relies on the environmental condition that an isolate is exposed to (Shen and Chou, 2016; Yang *et al*., 2016; van Teeseling *et al*., 2017). This was clearly illustrated by the results of this study. Isolates obtained are usually unicellular in nature; however, when isolated they exhibited filamentous-like morphology. These isolates may have undergone a morphological shift from their normal morphological state to filamentous-like possibly in response to changes in the environment they were exposed to. A variation in temperature, pH and nutrient concentration amongst others factors have been shown in previous studies to induce an intricate series of cellular events including changes in cellular morphology (Alonso *et al*., 2002 as cited by Khan *et al*., 2012). These factors may have contributed to stressful environment, which may have triggered a morphological change as a survival tactic.

Moreover, the use of morphological criteria to classify filamentous bacteria is an unreliable method for the measure of relatedness because organisms that phenotypically look the same may not be genetically related and *vice versa* (Ramothokang *et al.*, 2003; Martins *et al.*, 2004). This was in accordance with the results from this study where isolates that had different morphological features were genetically shown to be closely related. In addition, bacteria that are believed to be non-filamentous in nature were shown to exhibit morphological traits of filamentous bacteria. This was in agreement with the study by Marrengane (2007). Therefore, such findings clearly illustrate that microscopic characterisation alone can be misleading.

4.2. Bacteriophage isolation from activated sludge

Accurate identification of filamentous bacteria in AS is the first step to sludge bulking and foaming prevention as there are currently no effective control methods in place to mitigate this problem. One of the objectives of this study was to screen for bacteriophages which may be subsequently used to mitigate the overgrowth of filamentous bacteria. Since filamentous bacteria are responsible for sludge bulking and foaming, all isolates obtained from this study were used to screen for the presence of their co-occurring bacteriophages as a means to mitigate bulking and foaming. Screening of bacteriophages from wastewater treatment plants was performed using the spot test method, where isolated filamentous-like bacteria were used as hosts to isolate desired bacteriophages. Of the nine bacterial isolates, unfortunately none were shown to support plaque formation during incubation at different temperatures (37°C, 25°C), and times (48 hours, 24 hours) even after modifications. The experiments were conducted in quadruplet.

Successful application of phage therapy to wastewater treatment requires that a bacteriophage is isolated from the environment and shown to have certain characteristics including phage virulence, the inability to carry toxin genes and have the desirable host range (Withey *et al.*, 2005; Hyman *et al.*, 2019). Sixty percent of the bacteria isolated by Khan *et al.* (2006) from sludge samples were shown to support plaque formation.

There are various possible causes for the lack of phage isolation in this study in comparison to the high isolation in the study conducted by Khan *et al.* (2006). One possible cause could be the difference in the extraction methods used. Khan *et al.* (2002) included bacteriophage elution with beef extract solution, while in this study isolation was as per Khairnar (2014).

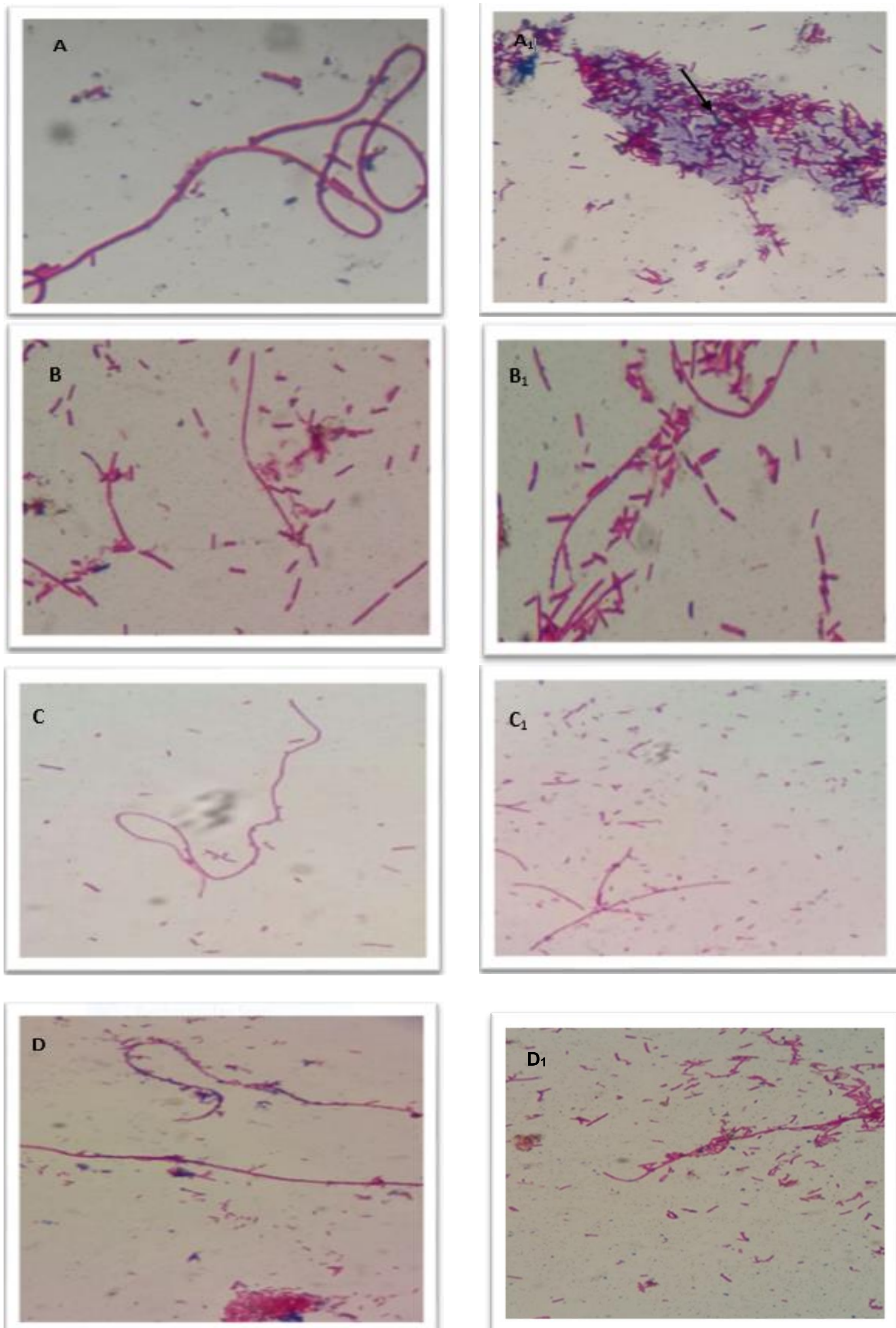


Figure 4.1: (A) Photo-micrograph of culture 11 (*Bacillus siamensis* 99.86%) before sub-culturing and (A1) after sub-culturing, (B) culture 7 (*Enterobacter ludwigii* 99.7%) before sub-culturing and (B1) after sub-culturing, (C) culture 19 (*Serratia marcescens subsp. Marcescens* 99.5%) before sub-culturing and (C1) after sub-culturing, (D) culture 17 (*Enterococcus faecalis* 99.9%) before sub-culturing and (D1) after sub-culturing

It has been shown that bacteriophages with broad host ranges are very common in AS (Hantula *et al*., 1991; Khan *et al*., 2002; Khan *et al*., 2006). This may have been one of the reasons why plaque formation was not observed in this study.

In addition to this, previous findings have shown that some bacteriophages fail to induce plaque formation from the original hosts from which they were isolated (Khan *et al*., 2006). This doesn't necessarily mean that infection does not occur; it is possible that bacteriophages infected the host bacteria and resulted in a lysogenic relationship. Based on the results by Khan *et al.* (2006) it was suggested that 92% of the bacterial isolates from AS environments were lysogens. Therefore, phage isolation for filamentous bacteria bulking control requires further investigation comparing different isolation methods.

Table 4.1: Isolates morphology and the percentage similarity of the sequences and the species level based on the 16S rRNA identification from EzBioCloud database

Isolate number	Gram stain	Morphological features	Species (Accession number)	Similarity
1	+	Long curved filament	Failed sequencing reaction	-
5	+	Long coiled filaments	*Bacillus siamensis* (KCTC 13613)	99.86%
6	+	Long curved filaments	Failed sequencing reaction	-
7	-	Short curved filaments	*Enterobacter ludwigii* (EN-119)	99.72%
9	+	Short straight filaments	Failed sequencing reaction	-
10	+	Long and short straight filaments	Failed sequencing reaction	-
11	+	Long curved filaments	*Bacillus siamensis* (KCTC 13613)	99.86%
17	+	Short curved filaments	*Enterococcus faecalis* (ATCC 19433)	99.93%
18	-	Long filaments with short filaments attached	*Klebsiella variicola* (DSM 15968)	99.79%
19	-	Curved filaments	*Serratia marcescens* subsp. *Marcescens* (ATCC 13880)	99.50%
20	-	Short straight filaments	*Serratia marcescens* subsp. *Marcescens* (ATCC 13880)	99.57%
21	-	Long Curved filaments	*Enterobacter ludwigii* (EN-119)	99.79%

Keys: + positive, - negative

CHAPTER 5

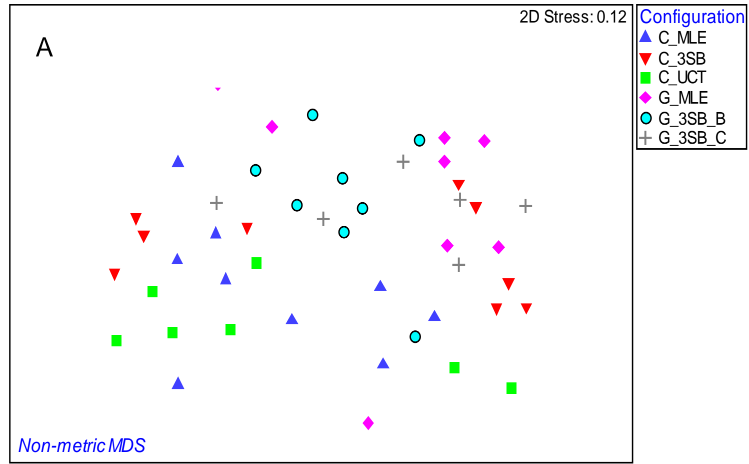
Next generation sequencing: Results and Discussion

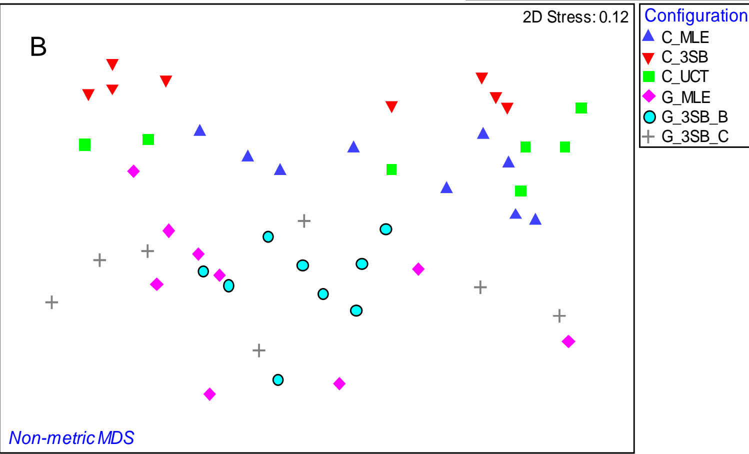
5.1. Analysis of the overall bacterial community structure in activated sludge

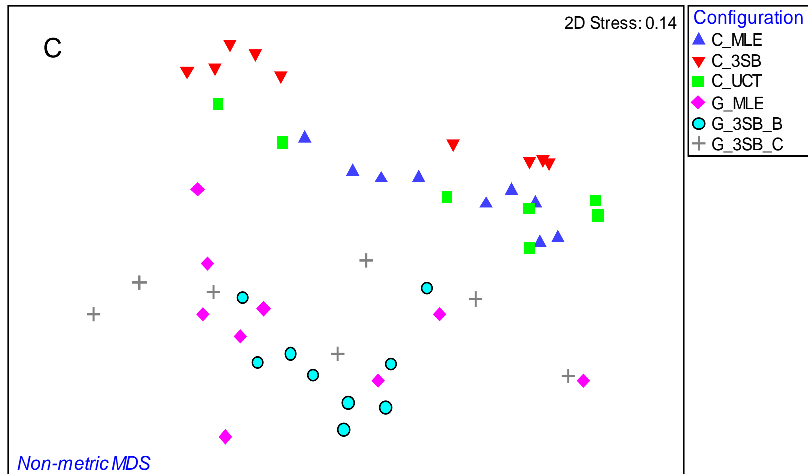
A total of 50 samples were taken from six plants with different reactor configurations, three of which were taken from Cape Town (Modified Ludzack-Ettinger, 3-Stage Bardenpho and University of Cape Town configuration) and the other three from Gauteng (Modified Ludzack-Ettinger,and two 3-Stage Bardenpho configuration). When the samples were originally taken, light microscope-based filament identification was performed according to the methods described by Eikelboom (2000) and Jenkins *et al.* (2004). The overall bacterial community structure in the AS from the study sites were determined using the results obtained from Next Generation Sequencing (NGS) performed using the Illumina (San Diego, USA) MiSeq workflow as per the manufacturer's guidelines at MR DNA (Shallowater, USA). The bacterial community similarity and diversity between samples were compared using Non-Metric Dimensional Scaling (nMDS) (ordination plots) as shown in Figure 5.1 to Figure 5.3 and the significance was further confirmed with ANOVA

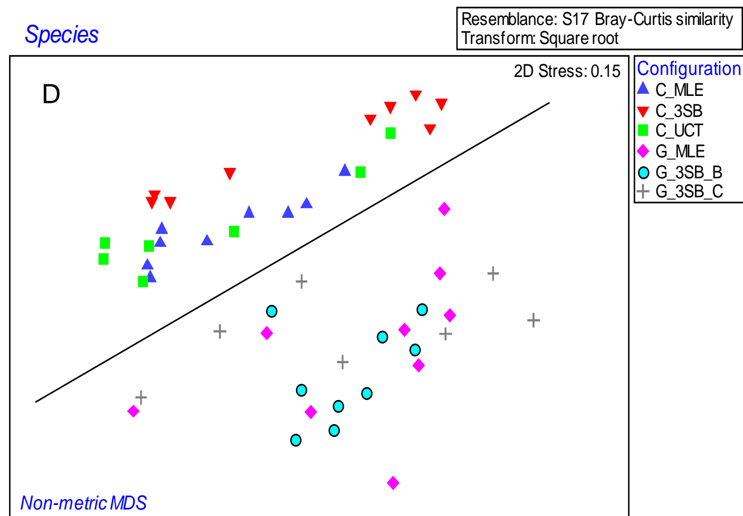
According to Bukin *et al.* (2018), some hypervariable regions have higher resolution for lower rank taxa (genera and species). This is in accord with the results of this study as shown by the ordination plots (Figure 5.1), which clearly show that clustering was more prominent at genus and species level (Figure 5.1C and Figure 5.1D, respectively) when compared with higher taxonomic levels (Figures 5.1A, 5.1B). The 2D stress values in the nMDS plots were 0.14 and 0.15 for lower taxonomic level, and 0.12 for higher taxonomic level.

NMDS ordination representing bacterial community revealed that at the species and genus level samples grouped according to the location (Figure 5.1C and Figure 5.1D) as all points representing the two geographic locations (CPT and GAU) are aligned on different sides of the plots as demarcated by the black line in Figure 5.1D. To determine the significance of geographic location on overall bacterial community structure, statistical analysis (ANOVA) was performed and revealed that geographic location was not a significant selective factor ($p > 0.05$) (Appendix 9)


Phylum (n=27)
Resemblance: S17 Bray-Curtis similarity
Transform: Square root
2D Stress: 0.12
A
Configuration
C_MLE
C_3SB
C_UCT
G_MLE
G_3SB_B
G_3SB_C
Non-metric MDS


Order (n=126)
Resemblance: S17 Bray-Curtis similarity
Transform: Square root
2D Stress: 0.12
B
Configuration
C_MLE
C_3SB
C_UCT
G_MLE
G_3SB_B
G_3SB_C
Non-metric MDS


Genus (n=831)
Resemblance: S17 Bray-Curtis similarity
Transform: Square root
2D Stress: 0.14
C
Configuration
C_MLE
C_3SB
C_UCT
G_MLE
G_3SB_B
G_3SB_C
Non-metric MDS



Legend

C_MLE – (Cape Town) Modified Ludzack-Ettinger

C_3SB – (Cape Town) 3-Stage Bardenpho configuration

C_UCT – (Cape Town) University of Cape Town

G_MLE – (Gauteng) Modified Ludzack-Ettinger

G_3SB_B – (Gauteng) 3-Stage Bardenpho configuration

G_3SB_C – (Gauteng) 3-Stage Bardenpho configuration

Figure 5.1: Non-metric multidimensional scaling plots of Bray-Curtis similarity of bacterial communities in selected wastewater treatment plants at phylum (A), order (B), genus (C) and species (D) taxonomic levels

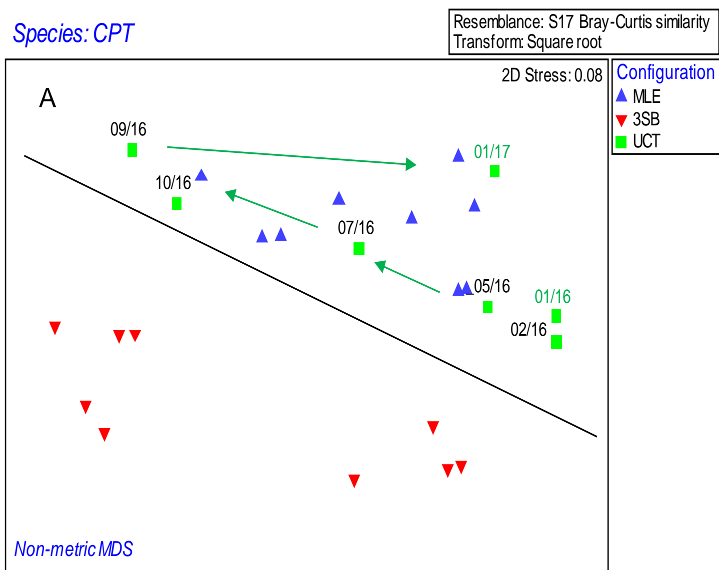
5.1.1. Comparison of the bacterial community structure in activated sludge samples from the City of Cape Town: effect of season and configuration

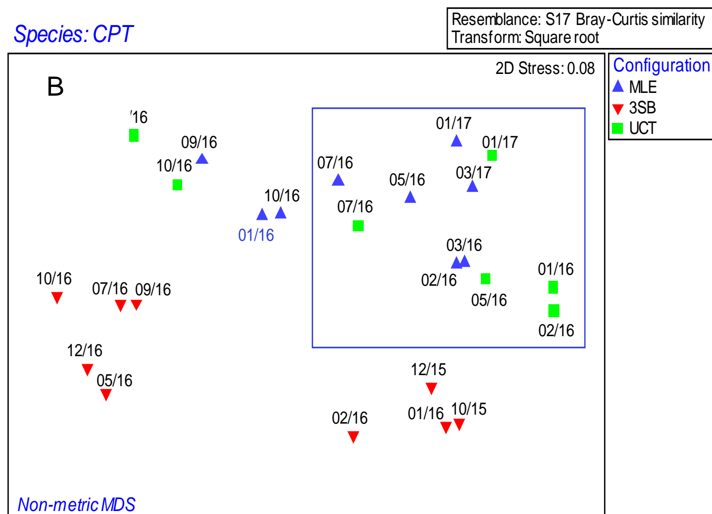
To ascertain whether there were differences in bacterial community structure in individual locations that were masked when the data was combined, the results from Cape Town were analysed separately from those of Gauteng. When CPT data was analysed separately, it was apparent that points representing MLE and UCT configured reactors clustered together. It is unlikely that this was linked to configuration because the UCT and 3SB configurations are highly similar. It may be because the 3SB reactor experienced severe bulking during this period. Based on statistical analysis reactor configuration was not a selective factor for bacterial community composition in CPT ($p>0.05$) (Appendix 9b)

Analysis of Figure 5.2, nMDS plots representing microbial community structure at different sampling times (summer, autumn, winter and spring) show that there was a

shift in microbial community composition during spring for MLE and UCT configured reactors. Points representing spring samples for these two configurations clustered closer to each other except for one point (in blue text) and away from the points representing summer, autumn and winter (blue triangle) (Figure 5.2B).

However, it is shown that points representing samples taken during the same month of successive years did not group closely to each other (Figure 5.2C). Rather samples taken in successive months tended to cluster closely to each other as shown in Figure 5.2A, where community shifts are denoted with green arrows.





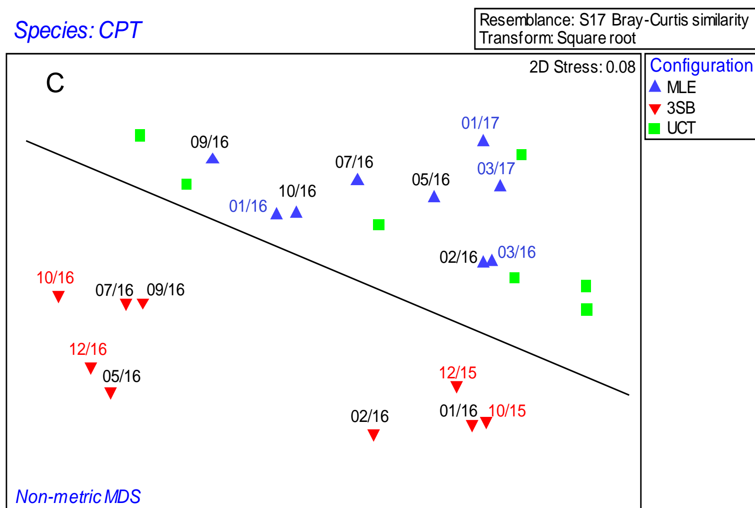
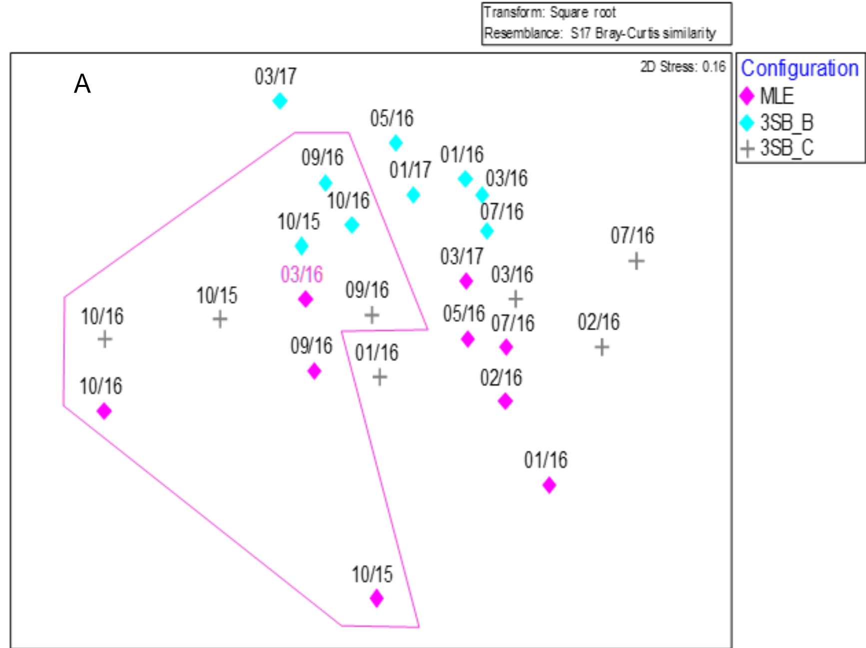


Figure 5.2: Non-metric multidimensional scaling plots of the Bray-Curtis similarity of bacterial communities in samples from the City of Cape Town showing shifts at successive months (A), seasonal shift (B), and successive years (C), the inserted numbers indicate the month and year in which the samples were taken

5.1.2. Comparison of the bacterial community structure in activated sludge samples from Gauteng: effect of season and configuration

Analysis of Figure 5.3, which represents the overall bacterial community composition from GAU reactor configurations reveals that reactor configuration did not appear to be the primary determinant of bacterial community composition. The points representing one of the 3SB configured WWTW (3SB_B) in the nMDS plots group away from the second 3SB configured WWTW (3SB_C) WWTW and MLE WWTW. MLE and 3SB_C configured WWTWs grouped together (Figure 5.3A-D). However, ANOVA showed that community composition from all three rector configurations was not significantly different (p>0.05) (Appendix 9c)

Figure 5.3A shows that there was a community shift, as points representing samples taken in spring clustered on one side of the plot (encased in pink). In contrast with CPT WWTWs, in the case of samples taken in successive years, some of the points clustered closely to each other especially those representing 3SB_B (in blue text) and 3SB_C (in grey text) Figure 5.3A and Figure 5.3B, respectively. This may be more of the relative stability of the bacterial structure in the WWTW than true seasonality.


Species: GAU
Non-metric MDS
Transform: Square root
Resemblance: S17 Bray-Curtis similarity
2D Stress: 0.16
A
Configuration
MLE
3SB_B
3SB_C
03/17
05/16
09/16
01/17
01/16
03/16
10/16
07/16
10/15
07/16
03/17
03/16
03/16
10/15
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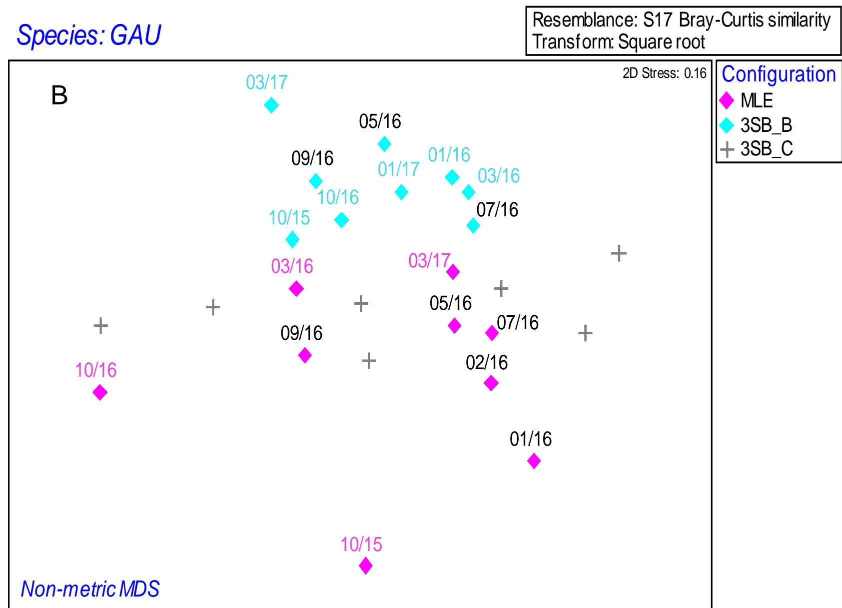
09/16
01/16
10/16
02/16
01/16
10/15


Species: GAU
Resemblance: S17 Bray-Curtis similarity
Transform: Square root
2D Stress: 0.16
B
Configuration
MLE
3SB_B
3SB_C
03/17
05/16
09/16
01/17
01/16
03/16
10/16
10/15
07/16
03/16
03/17
05/16
07/16
09/16
02/16
10/16
01/16
10/15
Non-metric MDS

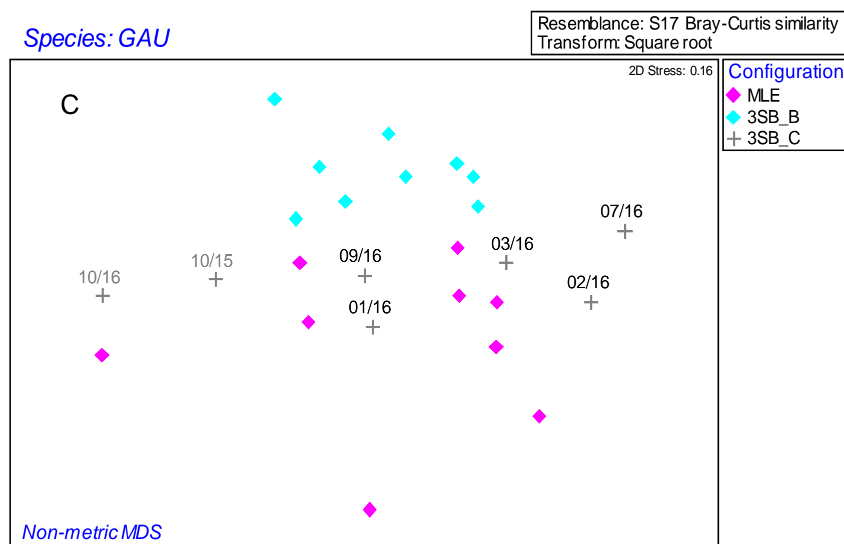


Figure 5.3: Non-metric multidimensional scaling plots of the Bray-Curtis similarity of bacterial communities in samples from Gauteng showing seasonal shift (A), 3SB_B shift at successive month (B), and 3SB_C shift at successive months (C) the inserted numbers indicate the month and year in which the samples were taken

5.2. Estimated absolute abundance of the dominant filamentous bacterial population

The main focus of this study was to evaluate filamentous bacteria in selected WWTWs. Filamentous bacteria identified through NGS were classified to their respective phyla and species group using the MIDAS 3 reference database classification system (available at https://www.midasfieldguide.org/guide/downloads; Accessed: 11November 2020). It was found that a diverse population of filamentous bacteria was present in the AS of the study WWTWs. Figure 5.5 is a representation of (A) estimated absolute abundance and (B) relative abundance of the filamentous genera and species identified, all of which fall under the phylum groups represented in Figure 5.4. The NGS results obtained were further compared with microscopy results obtained from a previous study using the same samples (Table 5.2).

A total of 27 phyla, 58 classes, 124 orders, 278 families, 832 genera and 1525 species of bacteria were identified. In order of dominance, the seven most abundant phyla were *Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Chloroflexi, Acidobacteria*, and *Planctomycetes* (Figure 5.4). These results are consistent with previous studies that determined the bacterial community structure in AS (Wang *et al*., 2012; Gao *et* al., 2016; Xu *et al*., 2018). *Proteobacteria*, which have the ability to degrade organic pollutants and remove nutrients such as nitrogen and phosphorus (Nielsen *et al*., 2010), was the most dominant phylum in all samples, irrespective of the geographic location and WWTW configuration. *Bacteroidetes*, the second most dominant phylum, plays an important role in wastewater treatment by degrading macromolecular organic

pollutants (Larsen *et al.*, 2008). *Actinobacteria* in wastewater participate in phosphorus removal and some genera can cause sludge bulking and foaming when present in excessive amounts (Saviour *et al.*, 2008). The phylum *Chloroflexi* is composed primarily of filamentous bacteria, which play a crucial role in sludge flocculation, providing a filamentous matrix around which desirable strong flocs with rapid settling properties are formed (Kragelund *et al.*, 2007; Speirs *et al.*, 2019).

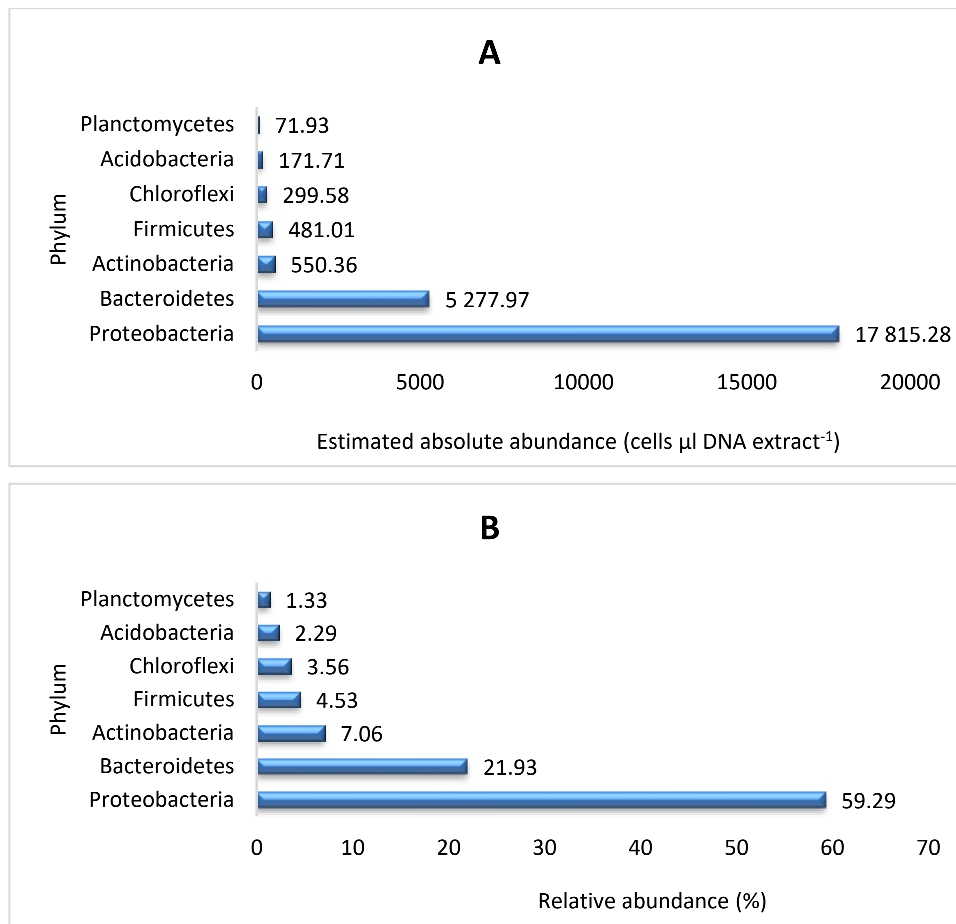


Figure 5.4: Estimated absolute abundance (cells µl DNA extract^{-1}) (A), and relative abundance (B) of the bacterial phyla in all six wastewater treatment plants

5.3. Dominant filamentous bacteria: comparison with microscopy findings, previous literature findings and association with geographic location, reactor configuration and seasonal variation

5.3.1. *Chloroflexi* filamentous bacteria

It has been shown that several filamentous bacteria in AS wastewater treatment plants across the world affiliate to the phylum *Chloroflexi* (Speirs *et al.*, 2019). *Chloroflexi* was one of the seven most dominant phyla in the AS samples analysed in this study, constituting on average 3.56% (0.30% - 9.92%) relative abundance and 3.00×10^2 (6.98×10^0 - 1.03×10^4) cells µl DNA extract^{-1} of the total reads across all WWTWs

(Figure 5.4A). The *Chloroflexi* classes *Caldilineae, Chloroflexia* and *Anaerolineae* made up the majority of the members of this phylum, this is consistent with a full-scale Danish survey by Nierychlo *et al.* (2018), in which 25 WWTWs were screened to identify the most important *Chloroflexi* genera using the FISH method. Most filamentous bacteria are classified up to genus level having no available cultured representatives and are given previously unpublished candidate names in the MIDAS database (Nierychlo *et al.*, 2020).

Amongst the *Chloroflexi* filamentous family group, morphotypes Eikelboom Type 0092 (*Ca Promineofilum*), Type 1851 (*Kouleothrix aurantiaca*) and Type 0041 are the most commonly encountered filamentous bacteria associated with bulking sludge in a number of studies carried out in AS WWTWs across the world, where microscopy was used as identification method (Speirs *et al.*, 2009; Welz *et al.*, 2014). This was in agreement with this study findings, however, all the MIDAs classified important genera that have been given provisional/candidate names were not detected by NGS. This was possibly due to the fact that different primers (V1-3 in MIDAs and V4 in this study) and database for taxonomic assignment (MIDAs based on SILVA and RDP II and NCBI in this study) were used. Thus, it is difficult to compare MIDAs and this study findings at lower taxonomic levels (Nierychlo *et al.*, 2020). For this study, it is therefore difficult to compare microscopy and NGS as identification methods using MIDAs as a reference for filamentous taxa. Eikelboom Type 0092 and 0803 filamentous bacteria are examples of this. Eikelboom Type 0092 filament was the most dominant filament identified in this study when using microscopy. However, *Ca Promineofilum* was not detected by NGS. Previous studies have only classified Type 0092 filament up to phylum level (Spiers *et al.,* 2009; Mielczarek *et al.*, 2012). Type 0092 isolates were never deposited in recognised culture collections. Thus, it is difficult to confirm their identity. The precise identification of Type 0092 claimed to have been cultured by Ramothokang *et al.* (2003) is unclear. According to Spiers *et al.* (2009), Type 0092 is not closely related to other described *Chloroflexi* filamentous bacteria found in AS, such as Eikelboom Type 1851 (*K. aurantiaca).* McIlroy *et al.* (2016) suggested that this filament may be a member of the class *Anaerolineae*, and in the absence of pure culture they proposed a provisional taxonomic assignment '*Candidatus Promineofilum breve'* (Nierychlo *et al.*, 2020). It is therefore important that an ecosystem specific database such as MIDAs be used to provide a common language for filament identification and avoid poor classification from large-scale public reference databases such as SILVA (Quast *et al.*, 2013), Greengenes (DeSantis *et al.*, 2006), and RDP (Cole *et al.*, 2013).

Just as Eikelboom Type 0092, Type 0803 is a common component of the AS community (Spiers *et al.*, 2015; Nierychlo *et al.*, 2019). As shown in Table 5.2, this morphotype constituted on average 2.4% of the total filamentous population identified microscopically in this study (Welz *et al.*, 2018). According to Kragelund *et al.* (2011) Type 0803 morphotype is associated with *Caldilinea* genus, while Speirs *et al.* (2015) suggested that Eikelboom Type 0803 is a member of the genus *Anaerolinea.* In this study estimated absolute quantification showed that *Anaerolinea* species constituted on average 6.77×10^{-1} (3.69×10^{-2} - 5.96×10^{0}) cells µl DNA extract^{-1} with relative abundance of 0.38% (0.02% - 3.72%) as shown in Figure 5.5. Although these filamentous bacteria were found in relatively low abundance, their growth was more favoured in GAU (Figure 5.6). This is in accordance with the microscopic findings for Type 0803 (Welz *et al.*, 2018). Figure 5.7 represents the abundance of different filamentous bacteria from all WWTWs reactor configurations combined (Figure 5.7A), CPT (Figure 5.7B) as well as GAU (Figure 5.7C) shows that 3SB configured reactors favoured the growth of *Anaerolinea* species. The same was observed for Type 0803 morphotype (Welz *et al.*, 2018). Based on the MIDAS 3 database classification, no morphotype has been associated with the *Anaerolinea* genus to date (Nierychlo *et al.*, 2020). Just as Type 0092, Type 0803 has been given a provisional candidate name '*Candidatus Defluviifilum*' yet to be incorporated into future versions of the MIDAS database (Nierychlo *et al.*, 2019). As *Candidatus Defluviifilum* is not yet included on the MIDAS database, it is impossible to compare NGS data with microscopy and further impossible to compare with previous studies.

Of the *Chloroflexi* filamentous bacteria, *K. aurantiaca* associated with Eikelboom Type 1851 morphotype has been grown and characterised phenotypically (Kohno *et al.*, 2002; Kragelund *et al.*, 2007). *K. aurantiaca* filamentous bacteria are found in most WWTWs, but generally in low abundance while Eikelboom Type 1851 morphotype dominates in most AS WWWs (Nierychlo and Nielsen, 2014; Nittami *et al.*, 2017) and is associated with filamentous bulking sludge (Kohno *et al.*, 2002; Nierychlo *et al.*, 2018; Spiers *et al.*, 2019; Nittami *et al.*, 2020). This is in agreement with this study findings, *K. aurantiaca* and other *Kouleothrix species* constituted on average 1.75% (0.01% - 40.55%) and 0.18% (0.01%-3.67%) of the total filamentous population respectively (Figure 5.5 B), while Eikelboom Type 1851 morphotype was the 5th most dominant constituting on average 11.9% of the total filamentous population using NGS and microscopy, respectively. Eikelboom Type 1851 morphotype was also found dominating in three filamentous surveys in South Africa (Blackbeard *et al.*, 1988; Lacko *et al.*, 1999; Welz *et al.*, 2014) and other countries (Beers *et al.*, 2002; Kragelund *et al.*, 2007; Nittami *et al.*, 2017). In the same samples analysed in this study, NGS findings

showed that *K. aurantiaca* and other *Kouleothrix* species were low in abundance compared to microscopy findings which showed that Eikelboom Type 1851 morphotype was among the most dominant filamentous bacteria. In addition, geographic location (site) and reactor configuration were not selecting factors for *K. aurantiaca* identified using NGS when data was combined (Figure 5.6 and Figure 5.7A). However, when reactor configurations from different sites were analysed separately it was shown that in the GAU reactors, *K. aurantiaca* was to some extent favoured in 3SB_C and MLE reactors in comparison to the 3SB reactors (Figure 5.7C), further suggesting that reactor configuration is not a selecting factor for *K. aurantiaca.* Findings based on microscopic identification showed that Eikelboom Type 1851 was more abundant in GAU. This suggests that not all Type 1851 filamentous bacteria are *K. aurantiaca,* this morphotype may be amongst those that embrace several phylogenetically very different bacteria, which are indistinguishable under the microscope (Speirs *et al.*, 2019). When determining the effect of seasonality on *Kouleothrix* species, it was shown that that they were less abundant during the colder seasons (Figure 5.8), which is in accordance with findings by Nittami *et al.* (2020).

Eikelboom Type 0041 is amongst the most commonly encountered morphotypes globally. It usually ranks highly in relative abundance in AS filament surveys (Jenkins *et al.*, 2004; Tandoi *et al.*, 2006; Seviour and Nielsen 2010; Speirs *et al.*, 2017; Welz *et al.*, 2018). This is in accordance with this study's microscopic findings, where this morphotype ranked as the 3rd most dominant morphotype. Previous FISH survey data suggested that Type 0041 is polyphyletic, i.e., it contains members which affiliate to different phyla (Bjornson *et al.*, 2002; Kagelund *et al.*, 2007; Mielczarek *et al.*, 2012; Nittami *et al.*, 2014). To date, this filament has not been cultured and there is no 16S or 23S rRNA sequence data available to elucidate its phylogeny (Speirs *et al.*, 2017; Speirs *et al.*, 2019). This morphotype is not associated with any genus/species currently on the MIDAS database (Nierychlo *et al.*, 2020).

According to MIDAS database classification, the genera *Bellilinea, Longilinea* and *Levilinea* are filamentous members of the *Chloroflexi* community. These were identified in this study through NGS and were amongst the most abundant filamentous bacteria. All three genera were not identified through microscopy and are not associated with any MIDAS morphotypes. Their abundance may suggest their importance in ASPs, therefore, they need to be studied further.

To determine the significance of geographic location, reactor configuration and seasonal variation on abundance of *Chloroflexi* filamentous bacteria, statistical analysis

(ANOVA) was performed and revealed that geographic location (site) was a significant ($p<0.05$) selective factor for the genera *Bellilinea*, and *Levilinea,* with their growth being favoured in CPT A (Appendix 5a). Reactor configuration was a selective factor for all *Chloroflexi* filamentous bacteria except *K. aurantiaca* ($p<0.05$ Appendix 5b). Seasonal variation was not a selective parameter for any filament within this group ($p>0.05$ Appendix 5c)

5.3.2. Actinobacteria

Members of this phylum are known to cause severe operational problems in AS (Nielsen *et al.*, 2009). These include well known *Candidatus Microthrix parvicella* (Rossetti *et al.*, 2005; Mielczerack *et al.*, 2012; Nierychlo *et al.*, 2020), *Mycolata* species and *N. limicola* II morphotypes now classified as several species in the genus *Tetrasphaera* (McKenzie *et al.*, 2006; Nguyen *et al.*, 2011). *M. parvicella*, a member of the genus *Ca Microthrix* is commonly found in AS plants (Martins *et al.*, 2004; McIlroy *et al.*, 2013; Fan *et al.*, 2019). Another filamentous member of this genus is *Candidatu*s *Microthrix calida*. According to Levantesi *et al.* (2006), this filament appears as a thinner version of *M. parvicella*, they only differ slightly in their trichome diameter. *M. parvicella* and *M. calida*, as well as *Gordonia paraffinivorans* were identified in this study together with filamentous-variable *Tetrasphaera jenkinsii*, *Tetrasphaera australiensis*, *Tetrasphaera vanveenii*, *Tetrasphaera* spp., *Mycobacterium* spp., and *Leifsonia* spp., as classified on the MIDAS database.

Most filamentous-variables were very low in abundance and were not identified through microscopy, and therefore, are not discussed further. An exception was *Tetrasphaera* spp. and *Mycobacterium* spp. *Tetrasphaera* spp. were the 3rd most dominant filament identified with relative and estimated absolute abundance of 9.8% (1.48%-28.85%) and 1.66×10^1 (1.60×10^0 - 7.62×10^1) cells μl^{-1} DNA extract (Figure 5.5A-B), according to MIDAS classification. *Tetrasphaera* species are associated with *N. limicola* morphotype which constituted on average 0.6% of the total filamentous population. *Tetrasphaera* spp. were highly abundant in CPT (Figure 5.6) in 3SB reactors (Figure 5.7A-C). The notable difference between *N. limicola* and *Tetrasphaera* spp. suggests that the latter is a novel species which may play a crucial role in AS judging from its abundance, and thus, requires further studying. *Mycobacterium* spp. was also amongst the most dominant filamentous bacteria in this study with estimated absolute abundance of 6.46×10^0 (5.46×10^{-1}- 7.62×10^1) cells μl DNA extract^{-1} and relative abundance of 3.3% (0.33% - 17.75%). *Mycobacterium* spp. were more abundant in CPT (Figure 5.6) in UCT configured reactors (Figure 5.7). When the reactor

configuration results were analysed separately, *Mycobacterium* spp. were still favoured in UCT configured CPT reactors and in both 3SB and MLE reactors in GAU.

Results based on microscopic identification showed that *M. parvicella* was the 2nd most dominant filament with average abundance of 16.5% (Table 5.2), contrary to NGS findings this filament was not as dominant it constituted 2.27% (0.17% - 10.45%) and estimated absolute abundance 4.37×10^{0} (2.35×10^{-1} - 3.36×10^{1}) cells µl DNA extract^{-1}. It can be suggested that not all the microscopy Eikelboom morphotypes identified were *Candidatus Microthrix parvicella*. In accordance with microscopic findings, geographic location was not a selective factor for *M. parvicella* (Figure 5.6). The same was observed for reactor configuration when results were combined. A similar trend was noted in the MLE and 3SB configured reactors, but abundance was slightly lower in the UCT reactor. When results were analysed separately, prevalence of *M. parvicella* was favoured in 3SB reactors in CPT and in MLE and 3SB_C reactors in GAU (Figure 5.7A-C).

To determine the significance of seasonality, geographic location and reactor configuration, ANOVA was performed and revealed that different seasons had no significant influence in dominance of all the filamentous bacteria of the phylum which includes *M. parvicella* ($p>0.05$ Appendix 6c). Geographic location was a significant parameter for selection of *Mycobacterium* spp. ($p<0.05$ Appendix 6b) and lastly, reactor configuration was a significant parameter for selection of *Tetrasphaera* spp. ($p<0.05$ Appendix 6b).

5.3.3. *Proteobacteria* filamentous bacteria

Proteobacteria is one of the phyla to which filamentous bacteria affiliate. Based on MIDAS database classification, there were a number of filamentous bacteria identified in this study as members of this group. Most were extremely low in abundance thus are not discussed further. *Sphaerotilus* spp. and *Candidatus Monilibacter batavus* are classified as filamentous-variable based on MIDAS database. *Sphaerotilus* spp. were the most dominant filamentous bacteria in this study and are members of the genus *Sphaerotilus* based on MIDAS database classification. A known morphotype affiliated to this genus is *Sphaerotilus natans,* commonly observed but not dominant in AS (Ramothokang *et al*., 2003). This was also observed in this study findings where microscopic identification was used (Welz *et al*., 2018). The genus *Sphaerotilus* spp. constituted 2.49×10^{1} (1.71×10^{0} - 8.03×10^{1}) cells µl DNA extract^{-1} with average relative abundance of 10.7% (0.91% - 24.31%) using NGS. However, the analysis was unable to discriminate to species level. Microscopic results showed that S. *natans* only

constituted 0.1% of the filamentous bacteriapopulation. It is possible that there are members of this genus not yet characterised in AS. *Sphaerotilus* spp., were most abundant in CPT (Figure 5.6) in UCT and MLE configured reactors, the same was observed when data from both locations were analysed separately (Figure 5.7 A-C). Figure 5.8A-C shows that *Sphaerotilus* spp. were more abundant in autumn. According to Liu, (2002), *S. natans,* just as Type 021N, is known to play a role in bulking sludge in industrial WWTWs.

Candidatus Monilibacter batavus was observed as being dominant by Levantesi *et al.* (2004) in industrial AS samples in Denmark, and the authors suggested that their dominance emphasises their importance in industrial WWTWs. This filament was not as dominant in this study (Figure 5.5). Its low abundance may be attributed to the fact that WWTWs included in this study treated primarily domestic influent. *M. batavus* was more abundant in CPT (Figure 5.6) in 3SB configured reactors (Figure 5.7). This filament is associated with the *N. limicola* morphotype which was the least dominant filament found with microscopy. Based on MIDAS classification, Type 021N morphotype is associated with *Thiothrix* species. It is worth mentioning that both *Thiothrix* and Type 021N morphotype were identified microscopically. However, when comparing the abundance of *Thiothrix* spp. with microscopy Type 021 findings, *Thiothrix* spp. were extremely low in abundance suggesting that not all Type 021N are *Thiothrix* filamentous bacteria. According to Kragelund *et al.* (2006) the morphology of Type 021N resembles that of *M. batavus.* Faheem and Khan (2009) showed that Type 021N was more abundant in warm temperatures, this was also observed for *M. batavus* which was more abundant in spring/summer (Figure 5.8).

Statistical analysis was performed to determine whether geographic location, reactor configuration and seasonal variation had a significant influence on selection of *Proteobacteria* filamentous bacteria and the results revealed that geographic location and reactor configuration were selecting factors for both *M. batavus* and *Sphaerotilus* spp. ($p<0.05$ Appendix 7A and Appendix 7B), while the opposite was true for seasonal variation for both filamentous bacteria ($p>0.05$ Appendix 7C).

5.3.4. ***Bacteroidetes*** **filamentous bacteria**

Following *Proteobacteria*, *Bacteroidetes* was the second most dominant phylum found in this study (Figure 5.4). Members of this group identified were filamentous *Haliscomenobacter* spp., *Haliscomenobacter hydrossis,* and *Lewinella* sp. In addition to these were the filamentous-variable *Fluviicola* spp., *Flavobacterium gelidilacus*, *Flavobacterium succinicans* and *Crocinitomix* spp. Most filamentous bacteria in this

phylum were not identified microscopically (Table 5.2), except *H. hydrossis.* There was no significant difference in abundance of *H. hydrossis* identified microscopically and through NGS (Table 5.2). In addition to *H. hydrossis* other members of the genus *Haliscomenobacter* were found. Both *Lewinella* and *Haliscomenobacter* spp. were more abundant in CPT with the exception of *H. hydrossis* which was more abundant in GAU (Figure 5.5) in 3SB and MLE reactors (Figure 5.7). Filamentous-variable *Fluviicola* spp., *Flavobacterium gelidilacus*, *Flavobacterium succinicans* and *Crocinitomix* are currently not associated with any morphotype, and thus, were not identified microscopically. Just as *H. hydrossis,* they were more abundant in GAU (Figure 5.5) in 3SB and MLE reactors (Figure 5.7). Statistical analysis revealed that location was a significant factor for dominance of *H. hydrossis, Crocinitomix* spp., and *Lewinella* sp. ($p<0.05$ Appendix 8A). Reactor configuration played a significant influence in selection of *Haliscomenobacter* spp., *H. hydrossis,* and *Lewinella* spp. Season was not a selective factor for any *Bacteroidetes* filamentous bacteria.

5.3.5. *Firmicutes* filamentous bacteria

Based on the MIDAs database, *Turicibacter sanguinis* is the only characterised filament within the phyum *Firmicutes* and was not identified in this study. However, uncharacterised *Turicibacter* spp., were identified in very low abundance.

5.3.6. Planctomycetes and Acidobacteria filamentous bacteria

No filamentous bacteria from the phylum *Acidobacteria* are currently found in the MIDAS database. *Planctomycetes* has only one characterised filament in the database (*Candidatus Nostocoida*), but it was not observed in this study.

Comparison of microscopy and NGS findings

Microscopy and NGS findings summarised in Table 5.2 highlight the difficulty of comparing morphotypes with taxonomic data obtained from amplicon sequencing. The current results and previous studies suggest that there may be multiple taxa constituting a particular morphotype and vice versa (Speirs *et al.*, 2019). Moreover, it was even difficult to compare taxonomy data from this study with the MIDAS database used as a reference database due to different methodologies used such as the choice of primer sets and database for taxonomic assignment. An alternative would be to also utilise Fluorescent in Situ Hybridization (FISH) which was also used in this study with limited success. The non-alignment of microscopy and NGS findings raises questions on accuracy of results where only microscopy is used especially for routine purposes. To ensure accurate classification, a holistic approach using both microscopy and molecular methods is recommended

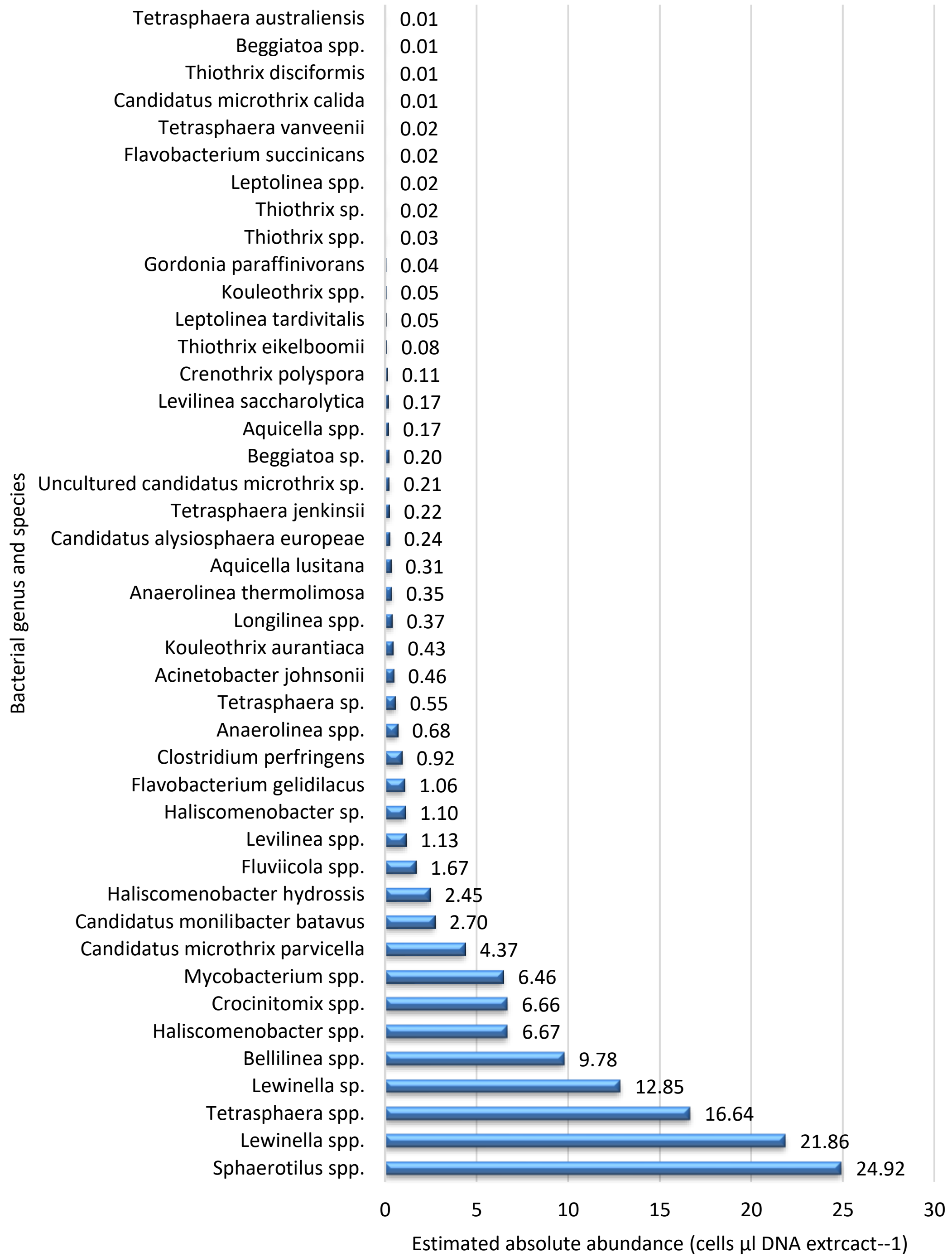
Table 5.2: Comparison of NGS and Microscopy findings

MIDAS genus/species name	Microscopic morpho-type	NGS results (cells µl DNA extract^{-1})	NGS results Relative abundance (%)	Microscopy results Relative abundance (%)
Chloroflexi				
Ca Promineofilum	Type 0092	ND	ND	28.6
-	Type 0041	ND	ND	14.1
Kouleothrix aurantiaca	Type 1851	4.30×10^{-1}	1.75	11.9
Kouleothrix spp.	-	4.69×10^{-2}	0.18	ND
-	Type 0803	2.56×10^{1}	10.3	2.4
Levilinea spp.	-	1.13×10^{0}	0.55	-
Anaerolinea spp.	-	6.77×10^{-1}	0.38	ND
Longilinea spp.	-	3.70×10^{-1}	4.91	ND
Actinobacteria				
Candidatus ***Microthrix Parvicella***	M. *Parvicella*	4.37×10^{0}	2.27	16.5
Candidatus ***Microthrix Calida***		1.41×10^{-2}	0	-
Uncultured *Candidatus* ***Microthrix*** sp.		2.08×10^{-1}	0.12	-
Gordonia paraffinivorans	*Gordonia amarae*-like organisms (GALO), *Nocardia amarae, Gordona*	3.84×10^{-2}	0.12	ND
Tetrasphaera spp.	*Nostcoida limicola* II	1.66×10^{1}	7.98	ND
Tetrasphaera sp.		5.50×10^{-1}	0.32	
Tetrasphaera jenkinsii		2.20×10^{-1}	0.13	
Tetrasphaera vanveenii		1.61×10^{-2}	0	
Tetrasphaera australiensis		1.01×10^{-2}	0	
Mycobacterium spp.	-	6.46×10^{0}	3.33	ND

Keys: ND not detected, - Not available

Table 5.2: ***Cont.*:**

Proteobacteria				
Sphaerotilus spp.	*S. natans*	2.49×10^{1}	10.7	0.1
Candidatus Monilibacter batavus	*N. limicola*	2.70×10^{0}	0.86	0.4
Thiothrix eikelboomii	Eikelboom Type 021N group I, II, III	8.04×10^{-2}	0.04	12.2
Thiothrix spp.		2.74×10^{-2}	0.01	
Thiothrix sp.		2.36×10^{-2}	0.01	
Thiothrix fructosivorans		4.60×10^{-3}	0.0	
Thiothrix disciformis		1.14×10^{-2}	0.01	
Aquicella lusitana	-	3.06×10^{-1}	0.23	-
Aquicella spp.	-	1.72×10^{-1}	0.04	-
Candidatus Alysiosphaera europeae	-	2.37×10^{-1}	0.08	-
Acinetobacter johnsonii	Type 1863	4.58×10^{-1}	0.33	ND
Bacteroidetes				
Haliscomenobacter hydrossis	*H. hydrossis*	2.45×10^{0}	0.33	0.9
Haliscomenobacter spp.	-	6.67×10^{0}	7.07	-
Haliscomenobacter sp.	-	1.10×10^{0}	0.61	-
Fluviicola spp.	-	1.67×10^{-0}	4.90	-
Lewinella sp.	-	1.28×10^{1}	0.89	-
Flavobacterium gelidilacus	-	1.06×10^{0}	0.97	-
Flavobacterium succinicans	-	2.13×10^{-2}		-
Crocinitomix spp.	-	6.66×10^{0}	2.81	-


A
Tetrasphaera australiensis 0.01
Beggiatoa spp. 0.01
Thiothrix disciformis 0.01
Candidatus microthrix calida 0.01
Tetrasphaera vanveenii 0.02
Flavobacterium succinicans 0.02
Leptolinea spp. 0.02
Thiothrix sp. 0.02
Thiothrix spp. 0.03
Gordonia paraffinivorans 0.04
Kouleothrix spp. 0.05
Leptolinea tardivitalis 0.05
Thiothrix eikelboomii 0.08
Crenothrix polyspora 0.11
Levilinea saccharolytica 0.17
Aquicella spp. 0.17
Beggiatoa sp. 0.20
Uncultured candidatus microthrix sp. 0.21
Tetrasphaera jenkinsii 0.22
Candidatus alysiosphaera europeae 0.24
Aquicella lusitana 0.31
Anaerolinea thermolimosa 0.35
Longilinea spp. 0.37
Kouleothrix aurantiaca 0.43
Acinetobacter johnsonii 0.46
Tetrasphaera sp. 0.55
Anaerolinea spp. 0.68
Clostridium perfringens 0.92
Flavobacterium gelidilacus 1.06
Haliscomenobacter sp. 1.10
Levilinea spp. 1.13
Fluviicola spp. 1.67
Haliscomenobacter hydrossis 2.45
Candidatus monilibacter batavus 2.70
Candidatus microthrix parvicella 4.37
Mycobacterium spp. 6.46
Crocinitomix spp. 6.66
Haliscomenobacter spp. 6.67
Bellilinea spp. 9.78
Lewinella sp. 12.85
Tetrasphaera spp. 16.64
Lewinella spp. 21.86
Sphaerotilus spp. 24.92
Bacterial genus and species
0
5
10
15
20
25
30
Estimated absolute abundance (cells µl DNA extrcact--1)

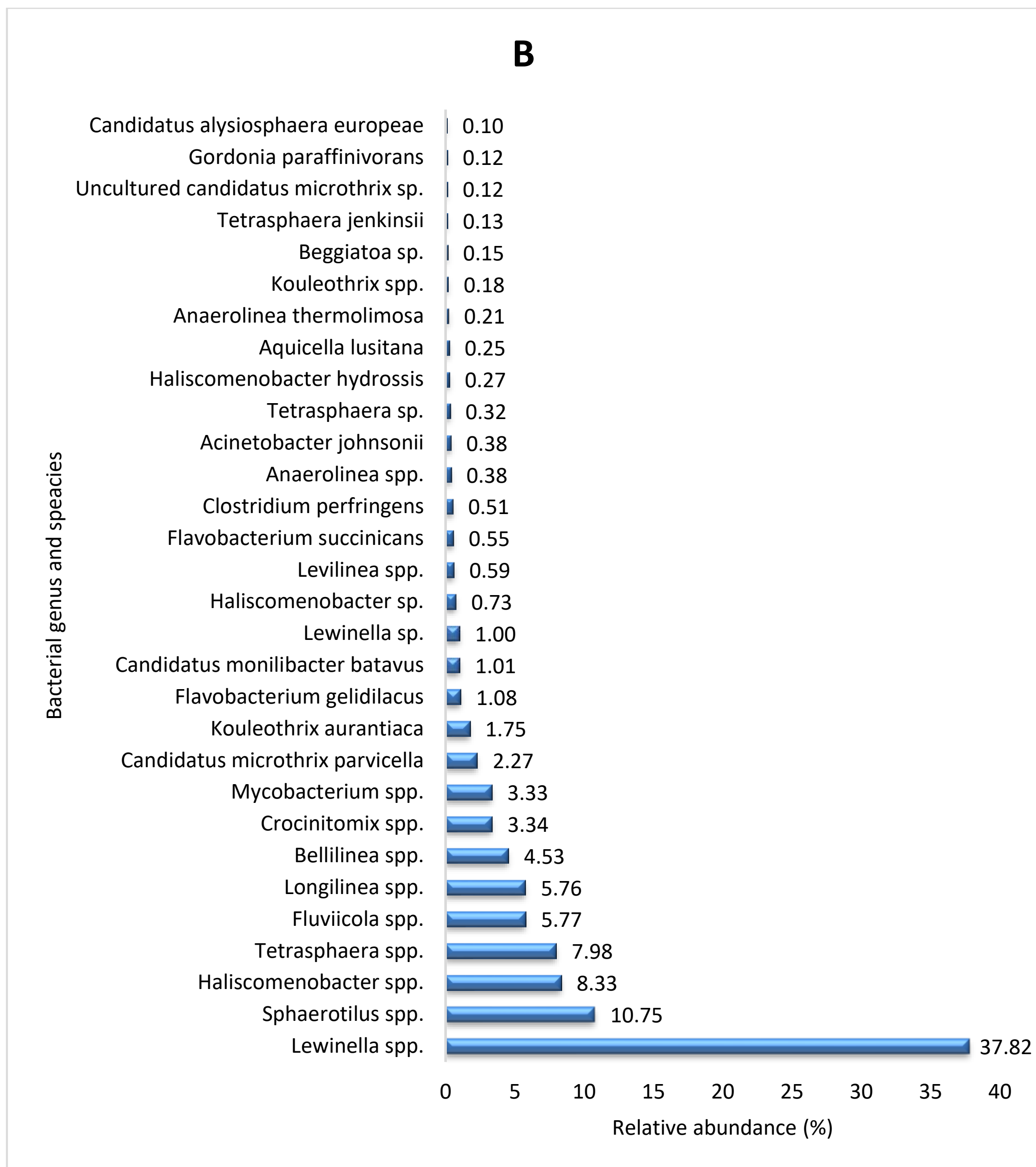


Figure 5.5: Estimated absolute abundance (A) and relative abundance (B) of the most dominant filamentous bacterial genus and species identified by amplicon sequencing from all samples from all wastewater treatment plants combined

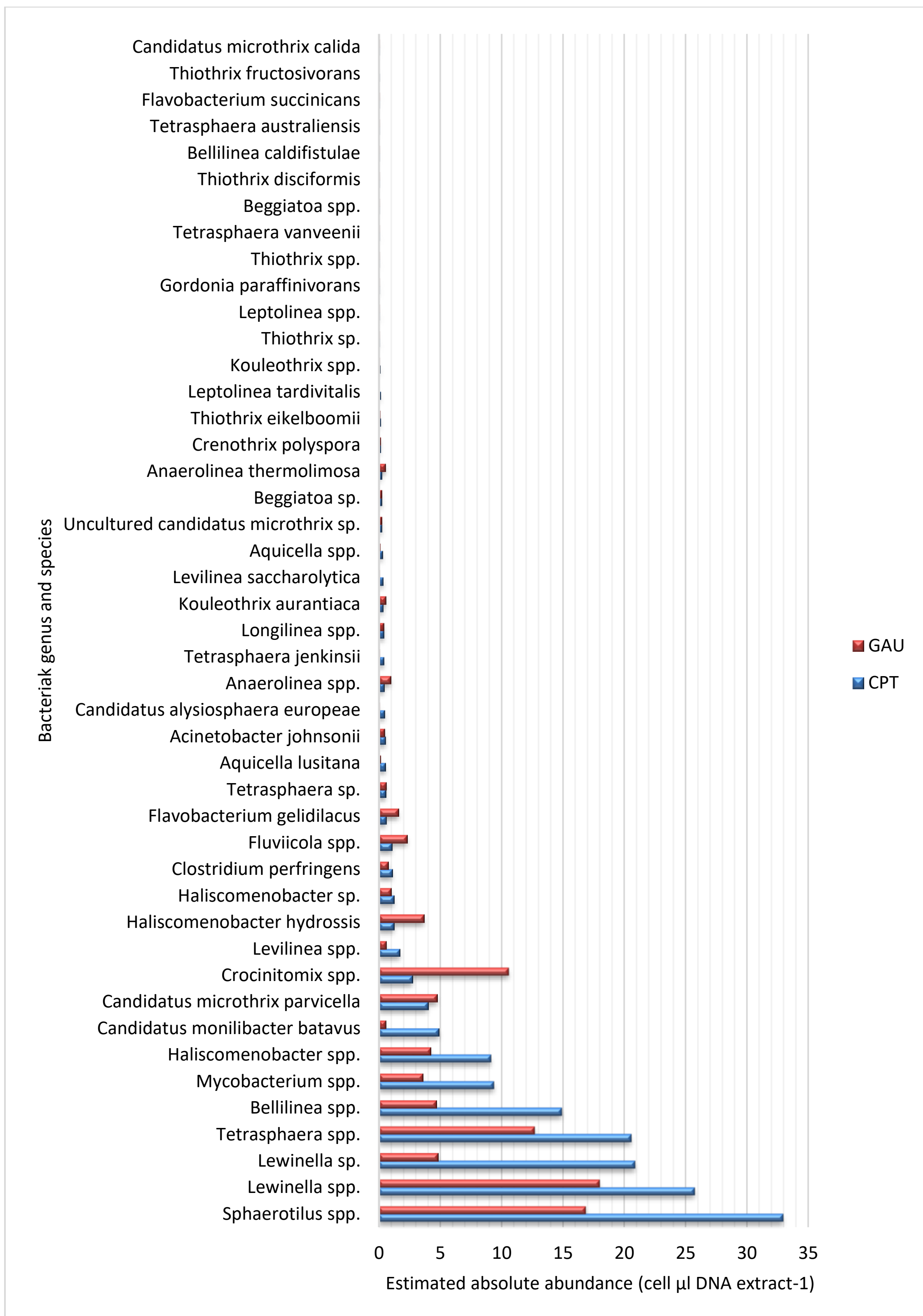
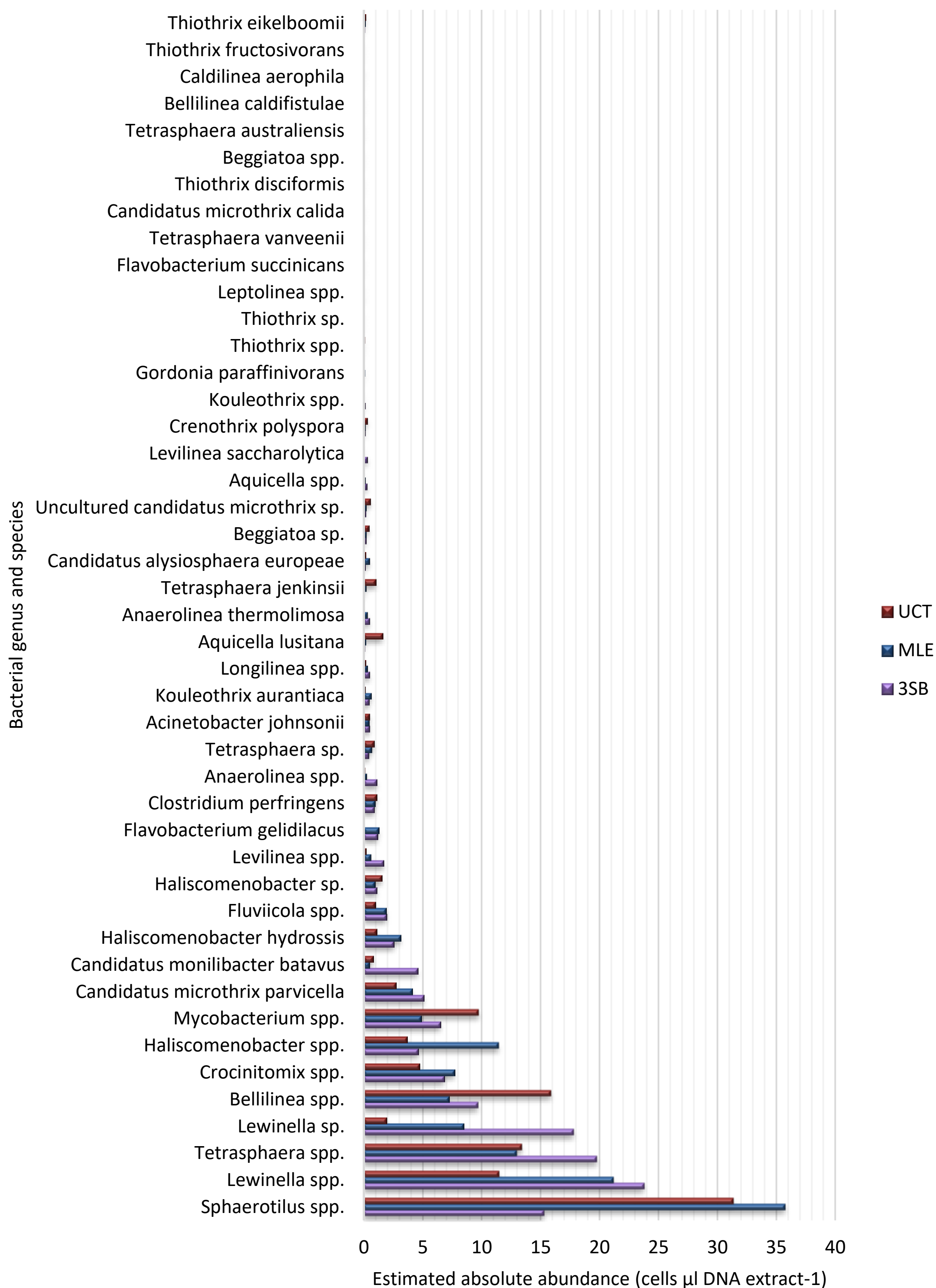


Figure 5.6: Estimated absolute abundance of the most dominant filamentous bacterial genus and species identified by amplicon sequencing from all samples from CPT and GAU wastewater treatment plants separated


A
Thiothrix eikelboomii
Thiothrix fructosivorans
Caldilinea aerophila
Bellilinea caldifistulae
Tetrasphaera australiensis
Beggiatoa spp.
Thiothrix disciformis
Candidatus microthrix calida
Tetrasphaera vanveenii
Flavobacterium succinicans
Leptolinea spp.
Thiothrix sp.
Thiothrix spp.
Gordonia paraffinivorans
Kouleothrix spp.
Crenothrix polyspora
Levilinea saccharolytica
Aquicella spp.
Uncultured candidatus microthrix sp.
Beggiatoa sp.
Candidatus alysiosphaera europeae
Tetrasphaera jenkinsii
Anaerolinea thermolimosa
Aquicella lusitana
Longilinea spp.
Kouleothrix aurantiaca
Acinetobacter johnsonii
Tetrasphaera sp.
Anaerolinea spp.
Clostridium perfringens
Flavobacterium gelidilacus
Levilinea spp.
Haliscomenobacter sp.
Fluviicola spp.
Haliscomenobacter hydrossis
Candidatus monilibacter batavus
Candidatus microthrix parvicella
Mycobacterium spp.
Haliscomenobacter spp.
Crocinitomix spp.
Bellilinea spp.
Lewinella sp.
Tetrasphaera spp.
Lewinella spp.
Sphaerotilus spp.
Bacterial genus and species
0
5
10
15
20
25
30
35
40
Estimated absolute abundance (cells µl DNA extract-1)
UCT
MLE
3SB

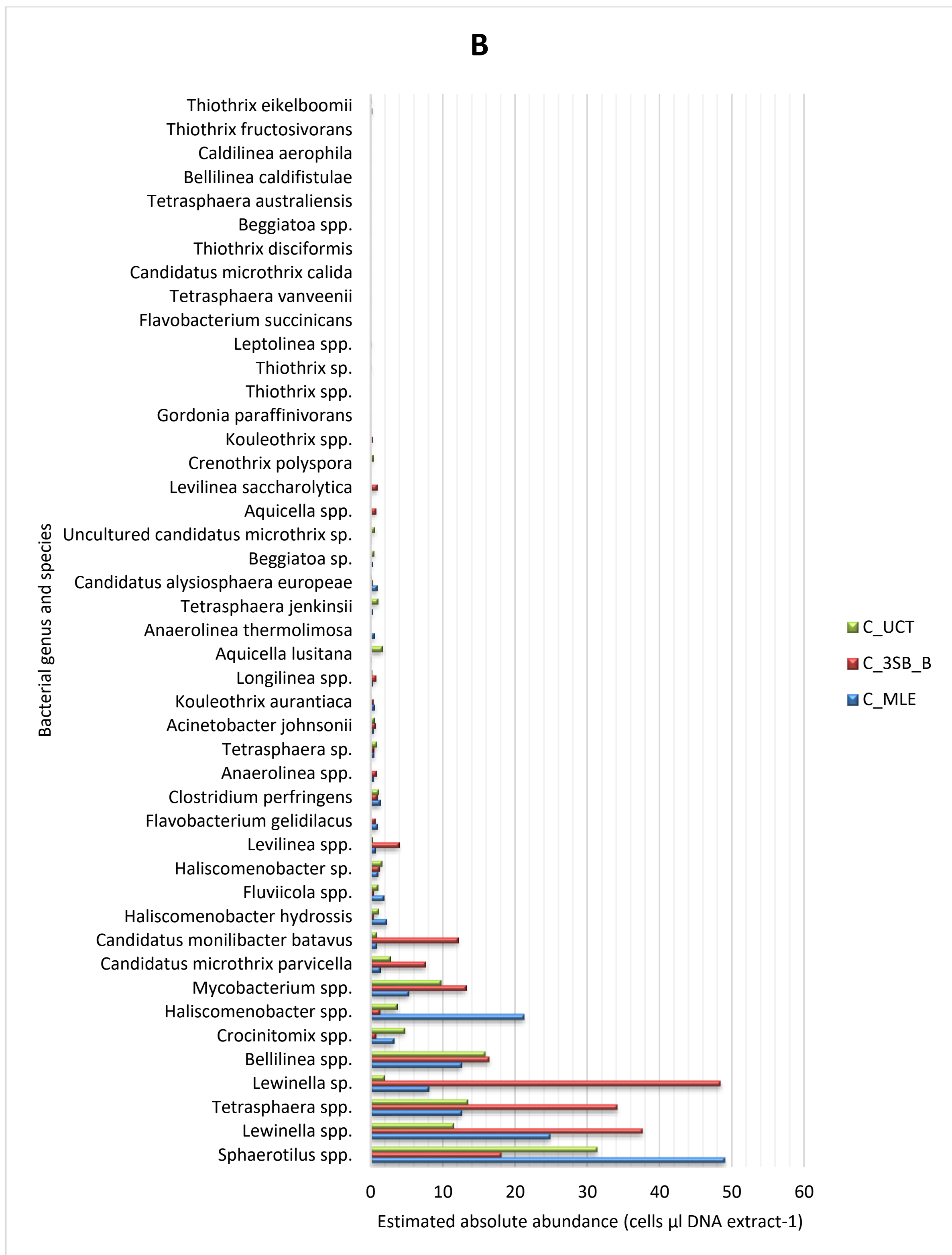
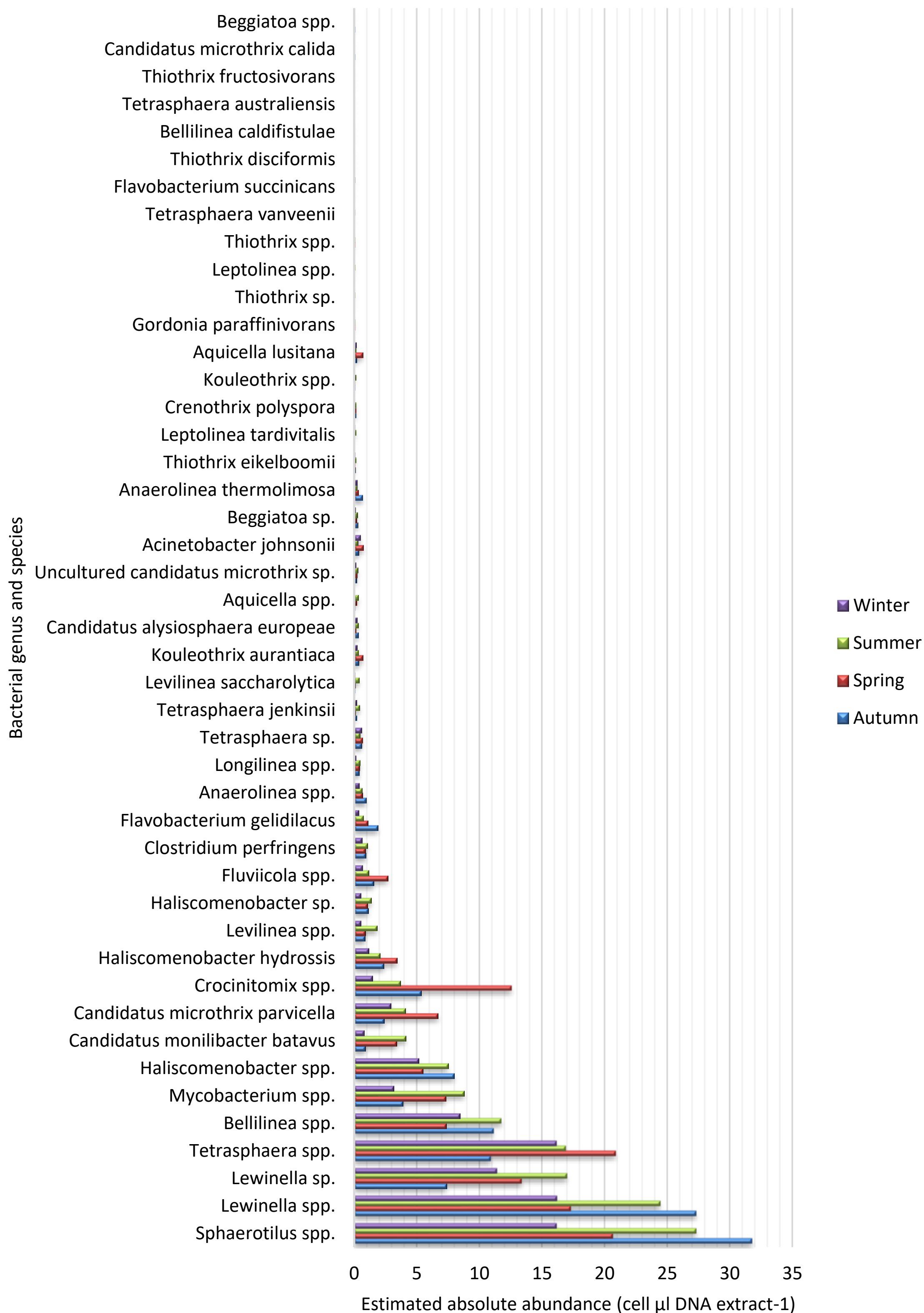


Figure 5.7: Estimated absolute abundant filamentous bacteria in all WWTWs with different process configurations from (A) both locations combined (B) CPT (C) GAU


A
Beggiatoa spp.
Candidatus microthrix calida
Thiothrix fructosivorans
Tetrasphaera australiensis
Bellilinea caldifistulae
Thiothrix disciformis
Flavobacterium succinicans
Tetrasphaera vanveenii
Thiothrix spp.
Leptolinea spp.
Thiothrix sp.
Gordonia paraffinivorans
Aquicella lusitana
Kouleothrix spp.
Crenothrix polyspora
Leptolinea tardivitalis
Thiothrix eikelboomii
Anaerolinea thermolimosa
Beggiatoa sp.
Acinetobacter johnsonii
Uncultured candidatus microthrix sp.
Aquicella spp.
Candidatus alysiosphaera europeae
Kouleothrix aurantiaca
Levilinea saccharolytica
Tetrasphaera jenkinsii
Tetrasphaera sp.
Longilinea spp.
Anaerolinea spp.
Flavobacterium gelidilacus
Clostridium perfringens
Fluviicola spp.
Haliscomenobacter sp.
Levilinea spp.
Haliscomenobacter hydrossis
Crocinitomix spp.
Candidatus microthrix parvicella
Candidatus monilibacter batavus
Haliscomenobacter spp.
Mycobacterium spp.
Bellilinea spp.
Tetrasphaera spp.
Lewinella sp.
Lewinella spp.
Sphaerotilus spp.
Bacterial genus and species
0
5
10
15
20
25
30
35
Estimated absolute abundance (cell µl DNA extract-1)
Winter
Summer
Spring
Autumn

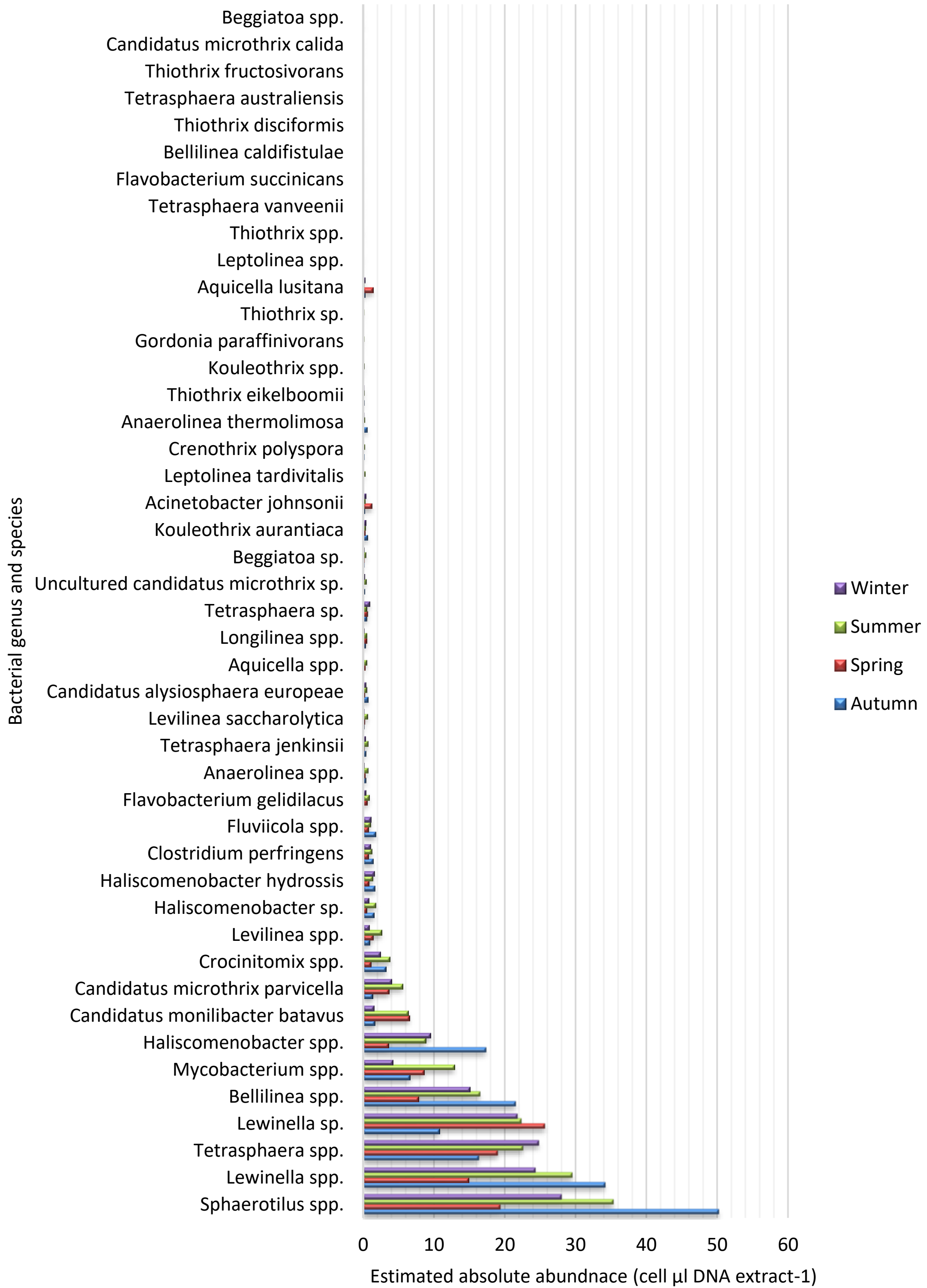

B
Beggiatoa spp.
Candidatus microthrix calida
Thiothrix fructosivorans
Tetrasphaera australiensis
Thiothrix disciformis
Bellilinea caldifistulae
Flavobacterium succinicans
Tetrasphaera vanveenii
Thiothrix spp.
Leptolinea spp.
Aquicella lusitana
Thiothrix sp.
Gordonia paraffinivorans
Kouleothrix spp.
Thiothrix eikelboomii
Anaerolinea thermolimosa
Crenothrix polyspora
Leptolinea tardivitalis
Acinetobacter johnsonii
Kouleothrix aurantiaca
Beggiatoa sp.
Uncultured candidatus microthrix sp.
Tetrasphaera sp.
Longilinea spp.
Aquicella spp.
Candidatus alysiosphaera europeae
Levilinea saccharolytica
Tetrasphaera jenkinsii
Anaerolinea spp.
Flavobacterium gelidilacus
Fluviicola spp.
Clostridium perfringens
Haliscomenobacter hydrossis
Haliscomenobacter sp.
Levilinea spp.
Crocinitomix spp.
Candidatus microthrix parvicella
Candidatus monilibacter batavus
Haliscomenobacter spp.
Mycobacterium spp.
Bellilinea spp.
Lewinella sp.
Tetrasphaera spp.
Lewinella spp.
Sphaerotilus spp.
Bacterial genus and species
0
10
20
30
40
50
60
Estimated absolute abundnace (cell µl DNA extract-1)
Winter
Summer
Spring
Autumn

Figure 5.8: Estimated absolute abundance of all filamentous bacteria in different weather seasons from both locations (A) combined, (b) CPT, (C) GAU

CHAPTER 6
Conclusions and Recommendations

6.1. Conclusions

This study aim was to identify Eikelboom type filamentous bacterial populations found in wastewater treatment plants in South Africa by means of both conventional (phenotypic) and molecular (phylogenetic) identification methods. Twelve isolates which exhibited filamentous morphology were successfully isolated, however, from the 16S rRNA gene analysis findings it was deduced that all the isolates were not Eikelboom type filamentous bacteria. From the findings it was concluded that isolation of Eikelboom Type filamentous bacteria is a difficult task. Fast growing non-filamentous bacteria tend to outgrow slow growing filamentous bacteria. In addition, non-filamentous bacteria tend change their morphology to filamentous form as a survival tactic. Moreover, it is likely that many filamentous morphotypes are either non-culturable or have more exacting growth requirements not previously elucidated. Consequently, attempts to isolate co-occurring bacteriophages were unsuccessful even with numerous repeated attempts. This may have been due to the fact that the method used was for isolation of Eikelboom type filamentous bacteria co-occurring bacteriophages, not the isolates obtained on this study.

Results based on NGS showed that the most dominant filamentous bacteria were novel species and were not previously identified via microscopy. An example was *Lewinella* sp. which was one ne of the most dominant filamentous bacteria identified by NGS and is not associated with any Eikelboom type morphotype. On the other hand, Eikelboom Type 0092 morphotype dominated during microscopic, but was not identified via NGS. From this alone it was deduced that comparing microscopy and NGS findings is challenging. Multiple taxa can constitute a particular morphotype and vice versa. It is apparent that there is a huge gap between the phylogeny and morphological approach with merits and disadvantages to each. For example, NGS is costly, however, offers detection with benefits with those added benefits being organism differentiation and novel organism discovery as opposed to microscopy. Microscopic identification on the other hand is rapid and cost-effective and more suited for routine purposes.

This study contributes to the body of knowledge on filament identification in the activated sludge of wastewater treatment plants from different geographical locations in South Africa as well as globally. The NGS findings from this study showed that there are many filamentous bacteria dominating the activated sludge plants and may be playing crucial roles which have not yet be studied and characterised, therefore this study can be used as a basis to gain further

knowledge on the phylogeny of the filamentous bacteria community, especially in the South African context.

6.2. Recommendations

As shown in this study, filament identification based on morphology as well as amplicon sequencing both have limitations. Research efforts to find more reliable, rapid and cost-effective molecular techniques for filament identification are required. Advances in micromanipulation techniques to definitively isolate single filamentous morphotypes and confirm their phylogeny and physiology should be sought. Moreover, the use of ecosystem-specific database (MIDAS) for activated sludge filamentous bacteria classification should be a norm.

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APPENDICES

APPENDIX 1: Growth media

Media name	Composition per litre	Grams
R2A	Agar	15.0g
	Yeast Extract	0.5g
	Casein	0.5g
	Glucose	0.5g
	Soluble starch	0.5g
	K_2HPO_4	0.3g
	Tryptone	0.25g
	Peptone	0.25g
	$MgSO_4$ anhydrous	0.024g
Glucose Yeast Extract	Agar	30g
	Peptone	10g
	Glucose	10g
	Yeast extract	5g
Actino YEME and supplement	Malt extract	10g
	Yeast extract	4g
	Glucose	4g
	Agar	20g
	Staph supplement	5ml

Medium preparation

Add all components to distilled water and bring volume to 1L. Mix thoroughly and autoclave at 121°C for 15 minutes.

APPENDIX 2: Gram stain method

Reagents

Solution A – Carbol fuchsin solution

Solution B – Iodine solution

Solution C – Alcohol solution

Solution D – Safranine solution

Method

- Prepare thin sample smears on microscope slides and allow to air dry
- Apply solution A for contact period of 1 minute, subsequently allow the excess dye to run off the slide
- Apply solution B for a contact period of 1 minute, subsequently allow excess dye to run off the slide
- Dip the slide in solution C for 30 seconds, move the slide to and fro in this solution.
- Rinse the slide clean with tap water by allowing the water to flow gently over the back of the slide.
- Apply solution D for a contact period of 120 seconds; subsequently rinse the slide again with tap water.
- Allow the slide to dry and view under microscope.

APPENDIX 3: DNA extraction protocol

Phosphate, SDS, Chloroform – Bead beater (PSC-B) method – modified for DNA extraction from actinobacteria

Reagents:

1. 100mM sodium phosphate buffer
   - For 100ml: Dissolve 1.199g NaH_2PO4 in 80ml dH_2O. Adjust pH to 8 (with NaOH pellets). Adjust to final volume of 100ml with dH_2O. Autoclave

2. SDS lysis buffer:
   - 100mM NaCl
   - 0.5M Tris-HCl (pH8)
   - 10% SDS
   - Add 8ml H_2O to 3g SDS, then add 15ml 1M Tris-HCl (pH8) and 0.6ml 5M NaCl. Heat gently to dissolve. Adjust final volume to 30ml with H_2O

3. Chloroform: isoamyl alcohol (24:1)

4. 7M Ammonium acetate
   - For 100ml: 53.956g ammonium acetate in 100ml dH_2O. Filter sterilize.

5. Isopropanol

6. 70% Ethanol

7. Bead-beater vials – i.e. 2ml screw capped, conical bottom, polypropylene tubes containing 0.5g each of 0.1mm and 3mm silica zirconium beads OR 0.5g seasand (Sigma) (i.e. quartz, qhite quartz sand, silicon dioxide).

Method:

1. Centrifuge down cell culture to collect cell pellet. Wash cell pellet with 1ml phosphate buffer (vortex). Spin at max speed for 5min. Remove supernatant.
2. Resuspend cell pellet in 300µl phosphate buffer and buffer+cells to bead-beater vial.
3. Add 300µl SDS lysis buffer, mix gently and then add 300µl chloroform:isoamyl alcohol. Mix gently and incubate at room temperature for 15minutes.
4. Vortex samples for 120s. Spin in microfuge at full speed (approx 15 000g or 13200rpm) for 5min to pellet cell debris.
5. Transfer the supernatant (approx 650µl) to a clean microfuge tube.

6. Add 7M ammonium acetate to achieve a final concentration of 2.5M (approx. 360µl).
7. Shake by hand to mix then spin at full speed for 5min. This should produce a clear supernatant. The cell debris should be extracted into the lower organic phase with the SDS and proteins forming a thick gel-like interface between the lower organic phase and the upper aqueous phase.
8. Transfer the supernatant (approx. 580µl) to a new tube. Add 0.54 volumes (315µl) isopropanol and incubate at room temperature for 2hrs. Spin at full speed for 5min.
9. Carefully remove supernatant and wash the pellet with 1ml 70% ethanol.
10. Spin at full speed for 5min. Remove supernatant and allow pellet to air dry (15-45min).
11. Resuspend DNA pellet in 100µl water or 10mM Tris-HCl (pH8) or TE buffer.

APPENDIX 4: Agarose gel electrophoresis

Solutions

1. 50X Tris Acetate Buffer (TAE 1L)
   - Weigh out 242g Tris-base and dissolve in approximately 700 ml deionised water
   - Carefully add 57.1 ml of acetic acid and 100 ml of 0.5M EDTA (pH 8)
   - Adjust the solution to 1L
2. Gel loading buffer
3. Electrophoresis buffer
   - 1X TAE buffer from 50X stock solution

Agarose gel

- Weigh out 1.5g agarose powder
- Dissolve with 100 ml 1X TAE buffer
- Microwave for 1 minute or until the solution becomes clear
- Allow to cool and add 1µl Ethidium bromide
- Mix gently
- Pour into a gel tray and carefully set the well comb in place
- Allow the gel to set for 15-20 minutes
- Remove the well comb and submerge the gel in electrophoresis buffer, ensure the gel is completely covered with buffer

Sample loading

- Mix 1 µl of gel loading buffer with 5 µl sample
- Carefully load the mixture in the wells
- Allow to electrophoresis at 100V for 1 hour

APPENDIX 5: Statistical analysis to determine the significance of geographic location, reactor configuration and seasonal variation on *Chloroflexi* filamentous bacteria selection

A. Geographic location: *Chloroflexi*

***Bellilinea* spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1308,705	1	1308,705	21,37809	2,87E-05	4,042652
Within Groups	2938,421	48	61,2171			
Total	4247,125	49				

***Longilinea* spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,005539	1	0,005539	0,018077	0,893608	4,042652
Within Groups	14,70629	48	0,306381			
Total	14,71183	49				

***Anaerolinea* spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3,805445	1	3,805445	2,71994	0,105632	4,042652
Within Groups	67,1564	48	1,399092			
Total	70,96185	49				

Kouleothrix aurantiaca

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,507243	1	0,507243	0,62634	0,432594	4,042652
Within Groups	38,87289	48	0,809852			
Total	39,38014	49				

***Levilinea* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	16,0904	1	16,0904	6,457885	0,014332	4,042652
Within Groups	119,5963	48	2,491589			
Total	135,6867	49				

B. Reactor configuration: *Chloroflexi* filament

***Bellilinea* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1501,827	5	300,3655	4,814079	0,00135	2,42704
Within Groups	2745,298	44	62,39314			
Total	4247,125	49				

Kouleothrix aurantiaca

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4,690332	5	0,938066	1,189829	0,329692	2,42704
Within Groups	34,6898	44	0,788405			
Total	39,38014	49				

***Anaerolinea* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	36,76211	5	7,352421	9,459326	3,54E-06	2,42704
Within Groups	34,19974	44	0,777267			
Total	70,96185	49				

Longilinea spp.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3,478743	5	0,695749	2,725248	0,03138	2,42704
Within Groups	11,23308	44	0,255297			
Total	14,71183	49				

Levilinea spp.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	87,78084	5	17,55617	16,1248	5,13E-09	2,42704
Within Groups	47,90581	44	1,088768			
Total	135,6867	49				

C. Seasonal variation: *Chloroflexi* filamentous bacteria

Bellilinea spp.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	189,4894	3	63,16312	0,716058	0,547464	2,806845
Within Groups	4057,636	46	88,20948			
Total	4247,125	49				

Anaerolinea spp.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1,505176	3	0,501725	0,332284	0,802024	2,806845
Within Groups	69,45667	46	1,509928			
Total	70,96185	49				

Kouleothrix aurantiaca

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	1,4451	3	0,4817	0,584109	0,628465	2,806845
Within Groups	37,93504	46	0,824675			
Total	39,38014	49				

***Longilinea* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0,529952	3	0,176651	0,57298	0,635662	2,806845
Within Groups	14,18187	46	0,308302			
Total	14,71183	49				

***Levilinea* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.810196	1	1.810196	1.232924	0.272371	4.042652
Within Groups	70.47425	48	1.468214			
Total	72.28444	49				

APPENDIX 6: Statistical analysis to determine the significance of geographic location, reactor configuration and seasonal variation on *Actinobacteria* filamentous bacteria selection

a. Geographic location:

Candidatus Microthrix parvicella

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7,272859	1	7,272859	0,183368	0,67041	4,042652
Within Groups	1903,803	48	39,66257			
Total	1911,076	49				

Mycobacterium spp.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	425,7869	1	425,7869	5,76354	0,020286	4,042652
Within Groups	3546,045	48	73,87594			
Total	3971,832	49				

***Tetrasphaera* spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	776,8914	1	776,8914	3,151713	0,082188	4,042652
Within Groups	11831,91	48	246,4981			
Total	12608,8	49				

b. Reactor configuration

Candidatus Microthrix parvicella

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	358,6384	5	71,72767	2,032943	0,092498	2,42704
Within Groups	1552,438	44	35,28268			
Total	1911,076	49				

Mycobacterium spp.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	762,4051	5	152,481	2,090456	0,084578	2,42704
Within Groups	3209,427	44	72,94152			
Total	3971,832	49				

***Tetrasphaera* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3460,013	5	692,0027	3,328104	0,012335	2,42704
Within Groups	9148,787	44	207,927			
Total	12608,8	49				

c. Seasonal variation

Candidatus Microthrix parvicella

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	148,0651	3	49,35504	1,287758	0,289841	2,806845
Within Groups	1763,011	46	38,32633			
Total	1911,076	49				

Mycobacterium spp.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	243,5844	3	81,19481	1,001801	0,400577	2,806845
Within Groups	3728,248	46	81,04886			
Total	3971,832	49				

Tetrasphaera **spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	683,6696	3	227,8899	0,879062	0,458919	2,806845
Within Groups	11925,13	46	259,242			
Total	12608,8	49				

APPENDIX 7: Statistical analysis to determine the significance of geographic location, reactor configuration and seasonal variation on *Proteobacteria* filamentous bacteria selection

a. Geographic location

Candidatus Monilibacter batavus

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	235,7802	1	235,7802	6,612815	0,013278	4,042652
Within Groups	1711,442	48	35,65505			
Total	1947,223	49				

***Sphaerotilus* spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3234,854	1	3234,854	8,258375	0,006027	4,042652
Within Groups	18801,88	48	391,7059			
Total	22036,74	49				

b. Reactor configuration

Candidatus Monilibacter batavus

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	972,2885	5	194,4577	8,776119	7,8E-06	2,42704
Within Groups	974,9342	44	22,1576			
Total	1947,223	49				

***Sphaerotilus* spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8050,666	5	1610,133	5,065459	0,000942	2,42704
Within Groups	13986,07	44	317,8652			
Total	22036,74	49				

c. Seasonal variation

Candidatus Monilibacter batavus

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	100,349	3	33,44966	0,833129	0,482572	2,806845
Within Groups	1846,874	46	40,14943			
Total	1947,223	49				

***Sphaerotilus* spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1404,78	3	468,26	1,04401	0,382094	2,806845
Within Groups	20631,96	46	448,5208			
Total	22036,74	49				

APPENDIX 8: Statistical analysis to determine the significance of geographic location, reactor configuration and seasonal variation on Bacteroidetes filamentous bacteria selection

Geographic location

Haliscomenobacter **spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	303,0229	1	303,0229	1,891196	0,175452	4,042652
Within Groups	7690,954	48	160,2282			
Total	7993,977	49				

Haliscomenobacter hydrossis

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	74,02202	1	74,02202	9,868562	0,002878	4,042652
Within Groups	360,0379	48	7,500791			
Total	434,06	49				

Crocinitomix **spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	771,0287	1	771,0287	4,204106	0,045809	4,042652
Within Groups	8803,149	48	183,3989			
Total	9574,178	49				

Fluviicola **spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18,85918	1	18,85918	3,119701	0,08371	4,042652
Within Groups	290,1691	48	6,045189			
Total	309,0283	49				

Flavobacterium gelidilacus

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	13,006	1	13,006	3,274678	0,076621	4,042652
Within Groups	190,6409	48	3,971686			
Total	203,6469	49				

Flavobacterium succinicans

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0,003022	1	0,003022	0,768493	0,38505	4,042652
Within Groups	0,188739	48	0,003932			
Total	0,191761	49				

***Haliscomenobacter* sp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0,659325	1	0,659325	0,911194	0,344581	4,042652
Within Groups	34,73201	48	0,723584			
Total	35,39134	49				

***Lewinella* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	748,8004	1	748,8004	2,042817	0,159683	4,051749
Within Groups	16861,43	46	366,5528			
Total	17610,23	47				

***Lewinella* sp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3224,38	1	3224,38	7,96317	0,006925	4,042652
Within Groups	19435,76	48	404,9116			
Total	22660,14	49				

Reactor configuration

***Haliscomenobacter* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2902,918	5	580,5835	5,017752	0,001008	2,42704
Within Groups	5091,06	44	115,7059			
Total	7993,977	49				

Haliscomenobacter hydrossis

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	122,9518	5	24,59035	3,477811	0,009808	2,42704
Within Groups	311,1082	44	7,070641			
Total	434,06	49				

***Crocinitomix* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	894,5416	5	178,9083	0,906947	0,485252	2,42704
Within Groups	8679,636	44	197,2645			
Total	9574,178	49				

***Fluviicola* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	65,93837	5	13,18767	2,387009	0,05323	2,42704
Within Groups	243,0899	44	5,52477			
Total	309,0283	49				

***Haliscomenobacter* sp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2,444838	5	0,488968	0,653016	0,660743	2,42704
Within Groups	32,9465	44	0,748784			
Total	35,39134	49				

***Lewinella* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	5401,959	5	1080,392	3,777779	0,00622	2,42704
Within Groups	12583,38	44	285,986			
Total	17985,34	49				

***Lewinella* sp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	14323,51	5	2864,701	15,11964	1,23E-08	2,42704
Within Groups	8336,632	44	189,4689			
Total	22660,14	49				

Flavobacterium gelidilacus

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	32,91773	5	6,583545	1,696699	0,155423	2,42704
Within Groups	170,7292	44	3,880209			

Total	203,6469	49				

Flavobacterium succinicans

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,027546	5	0,005509	1,476125	0,216965	2,42704
Within Groups	0,164215	44	0,003732			
Total	0,191761	49				

Seasonal variation

***Haliscomenobacter* spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	68,64837	3	22,88279	0,132816	0,940035	2,806845
Within Groups	7925,329	46	172,2898			
Total	7993,977	49				

Haliscomenobacter hydrossis

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	27,44409	3	9,148031	1,034907	0,386014	2,806845
Within Groups	406,6159	46	8,839476			
Total	434,06	49				

***Fluviicola* spp.**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	27,18858	3	9,062861	1,47918	0,232616	2,806845
Within Groups	281,8397	46	6,12695			
Total	309,0283	49				

***Haliscomenobacter* sp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3,247205	3	1,082402	1,548976	0,214602	2,806845
Within Groups	32,14413	46	0,698785			
Total	35,39134	49				

***Lewinella* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	988,7561	3	329,5854	0,891998	0,452439	2,806845
Within Groups	16996,59	46	369,491			
Total	17985,34	49				

***Lewinella* sp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	651,2821	3	217,094	0,453741	0,715907	2,806845
Within Groups	22008,86	46	478,4534			
Total	22660,14	49				

***Crocinitomix* spp.**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	885,2746	3	295,0915	1,562247	0,211334	2,806845
Within Groups	8688,903	46	188,8892			
Total	9574,178	49				

Flavobacterium gelidilacus

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	13,70931	3	4,569772	1,106729	0,356057	2,806845
Within Groups	189,9376	46	4,129079			
Total	203,6469	49				

Flavobacterium succinicans

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0,010746	3	0,003582	0,910296	0,443408	2,806845
Within Groups	0,181015	46	0,003935			
Total	0,191761	49				

APPENDIX 9: Statistical analysis to determine the significance of geographic location and reactor configuration on overall bacterial community structure

a. Geographic location

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4E-11	4	1E-11	1.22E-11	1	2.374071
Within Groups	3413.94	4155	0.821646			
Total	3413.94	4159				

b. Reactor Configuration (CPT)

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.71E-11	2	1.85E-11	2.83E-11	1	2.999335
Within Groups	1630.472	2493	0.65402			
Total	1630.472	2495				

c. Reactor Configuration (GAU)

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.41E-11	2	1.71E-11	1.93E-11	1	2.999335
Within Groups	2201.596	2493	0.883111			
Total	2201.596	2495				